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(54) Title: METHODS AND REAGENTS FOR ISOLATING BIOLOGICALLY ACTIVE ANTIBODIES

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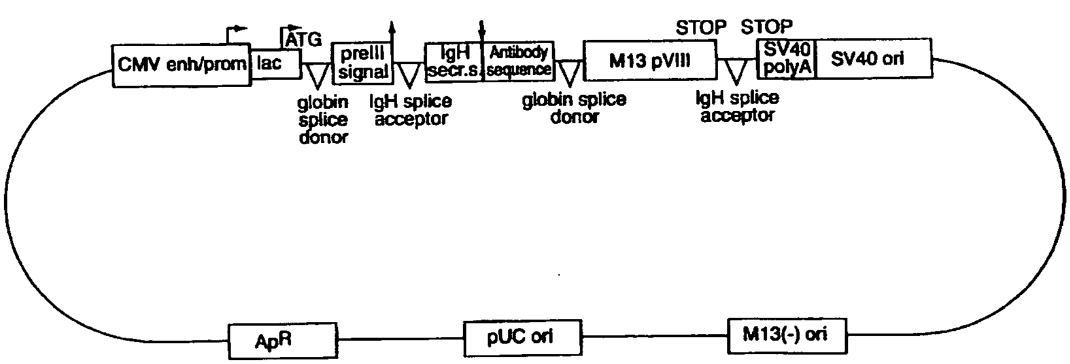

(57) Abstract: One aspect of the present invention is the synthesis of a binary method that combines variegated antibody display libraries, e.g., in a "display mode", with soluble secreted antibody libraries, e.g., in a "secretion mode", to yield a method for the efficient isolation of antibodies having a desired biological activity.

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Methods and Reagents for Isolating Biologically Active Antibodies

Background of the Invention

In an antibody-producing animal, such as a mammal, antibodies are synthesized and secreted into bodily fluids by plasma cells, a type of terminally differentiated B-lymphocyte. Exposure of the animal to a foreign molecule (i.e., via immunization) generally produces multiple plasma cell clones resulting in a heterogeneous mixture of antibodies (polyclonal antibodies) in the blood and other fluids. The blood of an immunized animal can be collected, clotted, and the clot removed to leave a serum containing the antibodies produced in response to immunization. This remaining liquid or serum, which contains the polyclonal antibodies, is referred to as antiserum. However, such antiserum contains many different types of antibodies that are specific for many different antigens. Even in hyperimmunized animals, seldom are more than one tenth of the circulating antibodies specific for the particular immunogen used to immunized the animal. The use of these mixed populations of antibodies, though useful in many situations, can create a variety of different problems in immunochemical techniques. For example, such antiserum will generally be inadequate for use in distinguishing between the immunogen and closely related molecules that share many common determinants with the immunogen.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler and Milstein (1975) *Nature* 256:495) represented a significant technical break-through with important consequences both scientifically and commercially. Monoclonal antibodies (MAbs) are traditionally made by isolating a single antibody secreting cell (e.g., a lymphocyte) from an immunized animal, fusing the lymphocyte with a myeloma (or other immortal) cell to form a hybrid cell (called a "hybridoma"), and then culturing the selected hybridoma cell *in vivo* or *in vitro* to yield antibodies which are identical in structure and specificity. Because the antibody-secreting cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The usefulness of monoclonal antibodies stems

While production of monoclonal antibodies has resulted in production of antibodies of greater specificity to a particular antigen than polyclonal methods, there are nevertheless a number of limitations associated with these techniques and antibodies produced thereby. For instance, a key aspect in the isolation of monoclonal antibodies relates to how many antibody-producing hybridoma cells with different specificities can be practically established and sampled in response to immunization with a particular antigen, compared to how many theoretically need to be sampled in order to obtain an antibody having specific characteristics. For example, the number of different antibody specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and represents only a small proportion of the potential repertoire of specificities. One technique that has emerged for identification of antibody leads involves the use of antibody display methodologies such as phage display. Phage-displayed antibody libraries can comprise vast collections of antibody variable regions that are displayed on the surface of a filamentous bacteriophage particle. Thus, each "antibody" is actually the N-terminal sequence of a phage-coat protein encoded by a randomly mutated region of the phage genome responsible for the production of the coat protein. In this manner, each unique antibody in the library is physically linked with the DNA molecule encoding it.

However, despite the success of these methods, they suffer from numerous sources of error and bias, such as very low initial concentrations of species, non-specific binding, and, significantly, the sampling of only a fraction of the library at the end of an experiment.

Summary of the Invention

One aspect of the invention provides a method for generating an antibody with a selected biological activity, comprising the steps of:

(i) providing an antibody display library comprising a variegated population of test antibodies expressed on the surface of a population of display packages;

(ii) in a display mode, isolating, from the antibody display library, a sub-population of display packages enriched for test antibodies which have a desired binding specificity and/or affinity for a cell or a component thereof;

(iii) in a secretion mode, simultaneously expressing the enriched test antibody sub-population under conditions wherein the test antibodies are secreted, dimerized, and are free of the display packages; and

(iv) assessing the ability of the secreted test antibodies to regulate a biological process in a target cell.

For instance, the antibody display library can be a phage display library, e.g., which utilizes phage particles such as M13, f1, fd, If1, Ike, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, ϕX-174, MS2 or f2. In preferred embodiments, the phage display library is generated with a filamentous bacteriophage specific for *Escherichia coli* and the phage coat protein is coat protein III or coat protein VIII. For instance, the filamentous bacteriophage can be M13, fd, and f1. In other embodiments, the antibody display library is a bacterial cell-surface display library or a spore display library.

In certain embodiments, the test antibodies are enriched from the antibody display library in the display mode by a differential binding means comprising affinity separation of test antibodies that specifically bind the cell or component thereof from test antibodies that do not. For example, the differential binding means can include panning the antibody display library on whole cells, affinity chromatographic means in which a component of a cell is provided as part of an insoluble matrix (e.g., a cell surface protein attached to a polymeric support), and/or immunoprecipitating the display packages.

In the display mode, the test antibodies can be enriched for those that bind to a cell-type specific marker and/or a cell surface receptor protein. For example, the test antibody library can be enriched in the display mode for test antibodies which bind to a G-protein coupled receptor, such as a chemoattractant antibody receptor, a neuroantibody receptor, a light receptor, a neurotransmitter receptor, a cyclic AMP receptor, or a polypeptide hormone receptor. In other embodiments, the test antibody

library can be enriched in the display mode for test antibodies that bind to a receptor tyrosine kinase, such as an EPH receptor. In still other embodiments, the test antibody library can be enriched in the display mode for test antibodies which bind to a cytokine receptor or an MIRR receptor.

In preferred embodiments, the antibody display library includes at least 10^3 different test antibodies.

In certain embodiments, each of the test antibodies are encoded by a chimeric gene comprising (i) a coding sequence for the test antibody, (ii) a coding sequence for a surface protein of the display package for displaying the test antibodies on the surface of a population of display packages, (iii) an antibody dimerization sequence, such as an Fc domain, and (iv) RNA splice sites flanking the coding sequence for the surface protein, wherein, in the display mode, the chimeric gene is expressed as fusion protein including the test antibody and the surface protein, whereas in the secretion mode, the test antibody is expressed without the surface protein as a result of the coding sequence for the surface protein being removed by RNA splicing.

In preferred embodiments, the test antibodies are expressed by a eukaryotic cell, more preferably a mammalian cell, in the secretion mode.

In preferred embodiments, the target cell is a eukaryotic cell, more preferably a mammalian cell such as a human cell.

In certain embodiments, the biological process scored for in the secretion mode includes a change in cell proliferation, cell differentiation or cell death. In other embodiments, the biological process that is detected is changes in intracellular calcium mobilization, intracellular protein phosphorylation, phospholipid metabolism, and/or expression of cell-specific marker genes.

In certain embodiments, the target cell includes a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the signal transduction acitivity of the cell surface receptor protein, expression of the reporter gene providing the detectable signal. For instance, the reporter gene can encode a gene product that gives rise to a detectable signal

selected from: color, fluorescence, luminescence, cell viability relief of a cell nutritional requirement, cell growth, and drug resistance. In preferred embodiments, the reporter gene encodes a gene product selected from chloramphenicol acetyl transferase, beta-galactosidase and secreted alkaline phosphatase. In other preferred embodiments, the reporter gene encodes a gene product that confers a growth signal. In certain embodiments, the secretion mode includes assessing the ability of the secreted test antibodies to inhibit the biological activity of an exogenously added compound on the target cells.

In an exemplary embodiment: in step (ii) above, display packages which bind to endothelial cells are isolated; and in step (iv) above, the ability of the secreted test antibodies to inhibit proliferation of endothelial cells is assessed. For example, in step (iv), the ability of the secreted, dimerized test antibodies to inhibit proliferation of endothelial cells in the presence of an angiogenic amount of an endogenous growth factor can be assessed.

The subject invention also specifically contemplates that antibodies identified in the secretion mode can be converted into peptidomimietics. Moreover, in certain embodiments, the subject method includes the further step of formulating, with a pharmaceutically acceptable carrier, one or more test antibodies which regulate the biological process in the target cell or peptidomimetics thereof. Another aspect of the present invention provides an antibody display library enriched for test antibodies having a desired binding specificity and/or affinity for a cell or a component thereof and which regulate a biological process in a target cell. Still another aspect of the present invention relates to a vector comprising a chimeric gene for a chimeric protein, which chimeric gene comprises (i) a coding sequence for a test antibody, (ii) a coding sequence for a surface protein of a display package, (iii) an antibody dimerization sequence, such as an Fc domain, and (iv) RNA splice sites flanking the coding sequence for the surface protein, wherein,
in a display mode, the chimeric gene is expressed as a fusion protein including the test antibody and the surface protein such that the test antibody can be displayed on the surface of a population of display packages, whereas in the secretion mode, the

test antibody is expressed without the surface protein, but fused with the antibody dimerization sequence, as a result of the coding sequence for the surface protein being removed by RNA splicing.

In certain embodiments, the chimeric gene can include a secretion signal sequence for secretion of the test antibody in the secretion mode, e.g., secretion of the test antibody from eukaryotic cells, preferably mammalian cells.

Yet another aspect of the present invention provides a vector library, each vector comprising a chimeric gene for a chimeric protein, which chimeric gene comprises (i) a coding sequence for a test antibody, (ii) a coding sequence for a surface protein of a display package, (iii) an antibody dimerization sequence, such as an Fc domain, and (iv) RNA splice sites flanking the coding sequence for the surface protein, wherein, in a display mode, the chimeric gene is expressed as fusion protein including the test antibody and the surface protein such that the test antibody can be displayed on the surface of a population of display packages, whereas in the secretion mode, the test antibody is expressed without the surface protein but as a fusion protein with the antibody dimerization sequence as a result of the coding sequence for the surface protein being removed by RNA splicing, the vector library collectively encodes a variegated population of test antibodies.

In preferred embodiments, the vector library collectively encodes at least 10^3 different test antibodies.

Another aspect of the present invention is a cell composition comprising a population of cells containing the vector library described above.

Still another aspect of the present invention provides a method for generating an antibody with a selected antimicrobial activity, comprising the steps of:

(i) providing a recombinant host cell population which expresses a soluble antibody library comprising a variegated population of test antibodies;

(ii) culturing the host cells with a target microorganism under conditions wherein the antibody library is secreted and diffuses to the target microorganism; and

(iii) selected host cells expressing test antibodies that inhibit growth of the target microorganism.

For example, the target microorganism is a bacteria or a fungus. In certain embodiments, the host cells are cultured on agar embedded with the target microorganisms. For example, antimicrobial activity of a test antibody can be determined by zone clearing in the agar.

Brief Description of the Drawings

Figure 1: Schematic of pAM6 M13/COS peptide expression plasmid.

Figure 2: Schematic of pAM7 & pAM9 M13/COS peptide expression plasmid.

Figure 3: Schematic of pAM8 M13/COS peptide expression plasmid.

Figure 4: Schematic of pAM7Fc, a plasmid similar to pAM7, except that in mammalian cells, it expresses the antibody Fc domain, which facilitates the dimerization of the V_H+V_L domains. In the pAM7Fc vector, the single chain V_H+V_L domains are expressed under the control of the *E. coli* lac promoter. The V_H+V_L domains are fused to an *E. coli* secretion sequence at their N-terminus and to the M13 pIII coat protein at their C-terminus. This arrangement ensures the packaging of the V_H+V_L-pIII fusion protein into the M13 capsid in the presence of an M13 helper phage. Upon transfection into COS cells, the plasmid is present in high copy number because of the presence of the SV40 origin of replication. The CMV enhancer/promoter drives the expression of the IgH heavy chain secretion signal sequence, the V_H+V_L domains, and the antibody Fc domain. The presence of the eukaryotic splice donor and acceptor sites results in the splicing out of the *E. coli* lac promoter, secretion signal sequence, and the M13 pIII protein. The resulting mRNA encodes the variable V_H+V_L domains linked to the Fc dimerization domain. Upon secretion the Fc domains dimerize resulting in a functional antibody dimmer.

Best Mode for Carrying Out the Invention

I. Overview

The present invention makes available a powerful directed approach for isolating biologically active antibodies. One aspect of the present invention is the

synthesis of a binary method that combines variegated antibody display libraries, e.g., in a "display mode", with soluble secreted antibody libraries, e.g., in a "secretion mode", to yield a method for the efficient isolation of antibodies having a desired biological activity.

Utilizing antibody display techniques, an antibody library can first be reduced in complexity by panning or other affinity purification techniques. In particular, the subject method selects antibodies having a certain affinity profile, e.g., a specificity and/or binding affinity for a discrete cell or protein or other cellular component thereof by (i) displaying the antibodies on the outer surface of a replicable genetic display package to create an antibody display library, and (ii) using affinity selection techniques to enrich the population of display packages for those containing antibodies which have a desired binding specificity for the target cell or cellular component (herein collectively referred to as the "target"). After the affinity enrichment step, the resulting sub-library is then utilized in a secretion mode whereby the test antibodies are secreted as soluble extracellular factors and their effect on cell phenotype or function is scored. In certain embodiments, a test antibody is fused to an antibody dimerization sequence, such as an Fc domain, to facilitate dimerization, which may increase biological activity of a test antibody. For instance, the ability of the secreted antibody to affect one or more of proliferation, differentiation, cell survival or cell death, secretion of growth factors or hormones, activation of gene transcription or the like can be detected. That is, the secretion mode measures biological activity of the test antibodies in order to distinguish between agonist, antagonist, and inactive antibodies with regard to regulating a particular biological response of a test cell or tissue.

In preferred embodiments, the display mode and secretion mode can be carried out without the need to sub-clone the test antibody coding sequence into another vector. To illustrate, Figures 1-4 show exemplary vectors for sequential use in both the display and secretion modes. In bacterial cells, the vectors produce a fusion protein consisting of a secretion signal sequence, the test antibody sequence and the remaining C-terminal portion of the gene pIII protein. The resulting chimeric

protein is capable of being incorporated into an M13 phage particle. However, in mammalian cells (such as COS cells), the M13 coding sequences are removed from the mature mRNA by virtue of splice sites which flank the phage sequence. Thus, the mature mRNA, in mammalian cells, encodes a secretion signal sequence and test antibody, optionally including an antibody dimerization sequence, which is secreted as a soluble (and optionally dimerized) antibody from the cell.

One advantage to such embodiments of the subject method is the ability to reduce loss of antibody sequences from the sub-library by eliminating sub-cloning steps.

In an exemplary embodiment, the subject method can be used to identify antibodies with anti-anigiogenic activity, e.g., the ability to reversibly inhibit proliferation of endothelial cells. In this regard, the present invention makes available a method for identifying endothelial inhibitors that can be used to inhibit angiogenesis-related diseases and modulating angiogenic processes. As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development, and formation of the corpus luteum, endometrium and placenta. The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels, and blood vessels. For instance, antibodies isolated by the subject method may be identified by their ability to bind to endothelial cells and overcome the angiogenic activity of endogenous growth factors such as bFGF, *in vitro.*

III. Definitions

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here. For the purpose of the present invention, the term "antibody" in its various grammatical forms is art-recognized and includes immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an

antigen. Structurally, the simplest naturally occurring antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. The light chains exist in two distinct forms called kappa (κ) and lambda (λ). Each chain has a constant region (C) and a variable region (V). Each chain is organized into a series of domains. The light chains have two domains, corresponding to the C region and the other to the V region. The heavy chains have four domains, one corresponding to the V region and three domains (1, 2 and 3) in the C region. The naturally occurring antibody has two arms (each arm being an Fab region), each of which comprises a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ from one antibody to another (owing to amino acid sequence variations). The variable domains for each of the heavy and light chains have the same general structure, including four framework regions (FRs), whose sequences are relatively conserved, connected by three hypervariable or complementarity determining regions (CDRs). The variable region of each chain can typically be represented by the general formula FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. The CDRs for a particular variable region are held in close proximity to one and other by the framework regions, and with the CDRs from the other chain and which together are responsible for recognizing the antigen and providing an antigen-binding site (ABS).

Moreover, it has been shown that the function of binding antigens can be performed by fragments of a naturally occurring antibody, and, as set out above, these antigen-binding fragments are also intended to be designated by the term "antibody". Examples of binding fragments encompassed within the term antibody include (i) the Fab fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) the Fd fragment consisting of the V_H and C_{H1} domains; (iii) the Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (iv) the dAb fragment (Ward et al., (1989) *Nature* 341:544-546) which consists of a V_H domain; (v) isolated CDR regions; and (vi) $F(ab')_2$ fragments, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region.

Furthermore, although the two domains of the Fv fragment are coded for by separate genes, it has proved possible to make a synthetic linker that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird et al. (1988) *Science* 242:423-426; and Huston et al. (1988) *PNAS* 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the present meaning of the term "antibody".

Finally, as will be evident from the disclosure herein, the subject method can be carried out using other binding domains, e.g., "antibody-like" polypeptide chains that provide a constant scaffold and one or more variable regions that participate in binding interactions of the molecule. For example, fibronectin can be used to create antibody-like polypeptides.

The language "antibody variable region" is likewise recognized in the art, and includes those portions of an antibody that can assemble to form an antigen binding site. For instance, an antibody variable region can comprise each of the framework regions (FR1-FR4) and complementary determining regions (CDR1-CDR3) for one or both chains of an IgG molecule.

The language "a desired binding specificity for an epitope", as well as the more general language "antibody specificity", refers to the ability of individual antibodies to specifically immunoreact with distinct antigens. The desired binding specificity will typically be determined from the reference point of the ability of the antibody to differentially bind, and therefore distinguish between, two different antigens - particularly where the two antigens have unique epitopes which are present along with many common epitopes. For instance, a desired binding affinity for an epitope can refer to the ability of an antibody to distinguish between related cells, such as between adult and fetal cells, or between normal and transformed cells. In other embodiments, the desired binding affinity can refer to the ability of the antibody to differentially bind a mutant form of a protein versus the wild-type protein, or alternatively, to discriminate in binding between different isoforms of a protein. An antibody that binds specifically to an epitope is referred to as a "specific antibody". The term "relative specificity" refers to the ratio of specific

immunoreactivity to background immunoreactivity (e.g., binding to non-target antigens). For instance, relative specificity for fetal cells can be expressed as the ratio of the percent binding to fetal cells to the percent binding to maternal cells. Antibody binding to antigen, though entirely non-covalent, can nevertheless be exquisitely specific for one antigen versus another, and often very strong. Antibodies can specifically bind different structural components of most complex protein, nucleic acid, and polysaccharide antigens. In general, macromolecules are much bigger than the antigen-binding site of an antibody. Therefore, an antibody binds to only a particular portion of the macromolecule, referred to herein as the "determinant" or "epitope". The total number of antibodies produced by a population of antibody-producing cells in a particular animal is referred to as the "antibody repertoire". The extraordinary diversity of the antibody repertoire is a result of variability in the structures of the antigen binding sites amongst the individual antibodies that make up the repertoire.

The language "variegated V-gene library" refers to a mixture of recombinant nucleic acid molecules encoding at least the antibody variable regions of one or both of the heavy and light chains of an antibody repertoire. The variable regions can be naturally occurring, e.g., cloned from B cells, or generated by random peptide sequences provided in place of one or more CDRs. A population of display packages into which the variegated V-gene library has been cloned and expressed on the surface thereof is likewise said to be a "variegated antibody display library" or "antibody display library".

The term "chimeric antibody" is used to describe a protein including at least the antigen-binding portion of an immunoglobulin molecule attached by peptide linkage to at least a part of another protein. A chimeric antibody can be, for example, an interspecies chimera, having a variable region derived from a first species (e.g., a rodent) and a constant region derived from a second species (e.g., a human), or alternatively, having CDRs derived from a first species and FRs and a constant region from a second species.

An antibody dimerization sequence refers to an amino acid sequence, preferably encodable by a nucleotide sequence, that forms a covalent or non-covalent association or complex with another like sequence. For example, constitutive dimerization is possible using leucine zipper or HLH (helix-loop-helix) domains, or any known homo-dimerization domain of known proteins, such as the C-terminal domain of lambda repressor. The leucine zipper contains a stretch of amino acids rich in leucine that are involved in dimerization of transcription factors. An adjacent basic region is responsible for binding to DNA. HLH (helix-loop-helix) proteins have amphipathic helices that are responsible for dimerization, adjacent to basic regions that bind to DNA. Alternatively, controllable dimerization is possible by selecting as a dimerization sequence D a sequence that binds a ligand L, and providing that ligand as a dimer L-L, thereby forming complexes of the form D~L-L~D. For example, two FK506-binding domains can be linked by using an FK506 dimer, as is well known in the literature. Analogously, a drug named AP1510 contains two FK506-like domains, each of which can bind to FKBP (FK506-Binding Protein), thus permitting the formation of complexes of the form D~LL~D. Peptide dimerization has been described in detail by Brennan et al., Oncogene 2000 Dec 11;19(53):6093-101; Alber, Curr. Opin. Genet. Dev. 1992 Apr;2(2):205-10; Garrell et al., Bioessays 1991 Oct;13(10):493-8; Wozney, Mol. Reprod. Dev. 1992 Jun;32(2):160-7; Zhang et al., Receptor 1993 Fall;3(3):183-91; Busch et al., Trends Genet. 1990 Feb;6(2):36-40; and Gibbons, Cell Motil. Cytoskeleton 1995;32(2):136-44.

The term “simultaneously expressing” refers to the expression of a representative population of an antibody library, e.g., at least 50 percent, more preferably 75, 80, 85, 90, 95 or 98 percent of all the different antibody sequences of a library.

The language “replicable genetic display package” or “display package” describes a biological particle that has genetic information providing the particle with the ability to replicate. The package can display a fusion protein including an antibody sequence derived from a variegated antibody library. The test antibody

portion of the fusion protein is presented by the display package in a context that permits the antibody to bind to a target that is contacted with the display package. The display package will generally be derived from a system that allows the sampling of very large variegated antibody libraries. The display package can be, for example, derived from vegetative bacterial cells, bacterial spores, and bacterial viruses.

The language "differential binding means", as well as "affinity selection" and "affinity enrichment", refer to the separation of members of the antibody display library based on the differing abilities of antibodies on the surface of each of the display packages of the library to bind to the target. The differential binding of a target by test antibodies of the display can be used in the affinity separation of those antibodies that specifically bind the target from those which do not. For example, the affinity selection protocol can also include a pre- or post-enrichment step wherein display packages capable of binding "background targets", e.g., as a negative selection, are removed from the library. Examples of affinity selection means include affinity chromatography, immunoprecipitation, fluorescence-activated cell sorting, agglutination, and plaque lifts. As described below, the affinity chromatography includes bio-panning techniques using either purified, immobilized target proteins or the like, as well as whole cells.

The phrases "individually selective manner" and "individually selective binding", with respect to binding of a test antibody with a target antigen, refers to the binding of an antibody to a certain protein target, which binding is specific for, and dependent on, the molecular identity of the protein target.

The term "solid support" refers to a material having a rigid or semi-rigid surface. Such materials will preferably take the form of small beads, pellets, disks, chips, dishes, multi-well plates, wafers or the like, although other forms may be used. In some embodiments, at least one surface of the substrate will be substantially flat. The term "surface" refers to any generally two-dimensional structure on a solid substrate and may have steps, ridges, kinks, terraces, and the like without ceasing to be a surface.

In an exemplary embodiment of the present invention, the display package is a phage particle that comprises an antibody fusion coat protein that includes the amino acid sequence of a test antibody. Thus, a library of replicable phage vectors, especially phagemids (as defined herein), encoding a library of antibody fusion coat proteins is generated and used to transform suitable host cells. Phage particles formed from the chimeric protein can be separated by affinity selection based on the ability of the antibody associated with a particular phage particle to specifically bind a target. In a preferred embodiment, each individual phage particle of the library includes a copy of the corresponding phagemid encoding the antibody fusion coat protein displayed on the surface of that package. Exemplary phage for generating the present variegated antibody libraries include M13, f1, fd, If1, Ike, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, φX-174, MS2 and f2.

The language "fusion protein" and "chimeric protein" are art-recognized terms which are used interchangeably herein, and include contiguous polypeptides comprising a first polypeptide covalently linked via an amide bond to one or more amino acid sequences which define polypeptide domains that are foreign to and not substantially homologous with any domain of the first polypeptide. One portion of the fusion protein comprises a test antibody, e.g., which can be random or semi-random. A second polypeptide portion of the fusion protein is typically derived from an outer surface protein or display anchor protein which directs the "display package" (as hereafter defined) to associate the test antibody with its outer surface. As described below, where the display package is a phage, this anchor protein can be derived from a surface protein native to the genetic package, such as a viral coat protein. Where the fusion protein comprises a viral coat protein and a test antibody, it will be referred to as an "antibody fusion coat protein". The fusion protein further comprises a signal sequence, which is a short length of amino acid sequence at the amino-terminal end of the fusion protein, that directs at least the portion of the fusion protein including the test antibody to be secreted from the cytosol of a cell and localized on the extracellular side of the cell membrane.

Gene constructs encoding fusion proteins are likewise referred to a "chimeric genes" or "fusion genes".

The term "vector" refers to a DNA molecule, capable of replication in a host cell, into which a gene can be inserted to construct a recombinant DNA molecule. The terms "phage vector" and "phagemid" are art-recognized and generally refer to a vector derived by modification of a phage genome, containing an origin of replication for a bacteriophage, and preferably, though optionally, an origin (*ori*) for a bacterial plasmid. The use of phage vectors rather than the phage genome itself provides greater flexibility to vary the ratio of chimeric antibody/coat protein to wild-type coat protein, as well as supplement the phage genes with additional genes encoding other antibody variable regions such as "auxiliary polypeptides" which may be useful in the "dual" antibody display constructs described below. The language "helper phage" describes a phage which is used to infect cells containing a defective phage genome or phage vector and which functions to complement the defect. The defect can be one that results from removal or inactivation of phage genomic sequence required for production of phage particles. Examples of helper phage are M13K07.

As used herein, "cell surface receptor" refers to molecules that occur on the surface of cells, interact with the extracellular environment, and (directly or indirectly) transmit or transduce the information regarding the environment intracellularly in a manner that may modulate intracellular second messenger activities or transcription of specific promoters, resulting in transcription of specific genes.

As used herein, "extracellular signals" include a molecule or other change in the extracellular environment that is transduced intracellularly via cell surface proteins that interact, directly or indirectly, with the signal. An extracellular signal or effector molecule includes any compound or substance that in some manner alters the activity of a cell surface protein. Examples of such signals include, but are not limited to, molecules such as acetylcholine, growth factors and hormones, lipids,

sugars and nucleotides that bind to cell surface and/or intracellular receptors and ion channels and modulate the activity of such receptors and channels.

As used herein, "extracellular signals" also include as yet unidentified substances that modulate the activity of a cellular receptor, and thereby influence intracellular functions. Such extracellular signals are potential pharmacological agents that may be used to treat specific diseases by modulating the activity of specific cell surface receptors.

"Orphan receptors" is a designation given to a receptors for which no specific natural ligand has been described and/or for which no function has been determined.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control sequences can be directly or indirectly regulated by the target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

The term "indicator gene" generically refers to an expressible (e.g., able to be transcribed and, optionally, translated) DNA sequence that is, for example, expressed in response to a signal transduction pathway modulated by a target receptor or ion channel. Exemplary indicator genes include unmodified endogenous genes of the host cell, modified endogenous genes, or a reporter gene of a heterologous construct, e.g., as part of a reporter gene construct.

"Signal transduction" is the processing of physical or chemical signals from the cellular environment through the cell membrane, and may occur through one or more of several mechanisms, such as activation/inactivation of enzymes (such as proteases, or other enzymes which may alter phosphorylation patterns or other post-translational modifications), activation of ion channels or intracellular ion stores, effector enzyme activation via guanine nucleotide binding protein intermediates,

formation of inositol phosphate, activation or inactivation of adenylyl cyclase, direct activation (or inhibition) of a transcriptional factor and/or activation.

The term "modulation of a signal transduction activity of a receptor protein" in its various grammatical forms, as used herein, designates induction and/or potentiation, as well as inhibition of one or more signal transduction pathways downstream of a receptor.

Agonists and antagonists are "receptor effector" molecules that modulate signal transduction via a receptor. Receptor effector molecules are capable of binding to the receptor, though not necessarily at the binding site of the natural ligand. Receptor effectors can modulate signal transduction when used alone, i.e., can be surrogate ligands, or can alter signal transduction in the presence of the natural ligand, either to enhance or inhibit signaling by the natural ligand. For example, "antagonists" are molecules that block or decrease the signal transduction activity of receptor, e.g., they can competitively, non-competitively, and/or allosterically inhibit signal transduction from the receptor, whereas "agonists" potentiate, induce, or otherwise enhance the signal transduction activity of a receptor. The terms "receptor activator" and "surrogate ligand" refer to an agonist that induces signal transduction from a receptor.

The term "compound" as used herein is meant to include both exogenously added test compounds and antibodies expressed from an antibody library.

III. Exemplary Embodiments

A. Display Mode

In its "display mode", a library of test antibodies is expressed by a population of display packages to form an antibody display library. With respect to the display package on which the variegated antibody library is manifest, it will be appreciated from the discussion provided herein that the display package will preferably be able to be (i) genetically altered to encode heterologous antibody, (ii) maintained and amplified in culture, (iii) manipulated to display the antibody-containing gene product in a manner permitting the antibody to interact with a target during an affinity separation step, and (iv) affinity-separated while retaining the

nucleotide sequence encoding the test antibody (herein "antibody gene") such that the sequence of the antibody gene can be obtained. In preferred embodiments, the display remains viable after affinity separation.

Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages or further manipulation of that sequence in the secretion mode. The most attractive candidates for this type of screening are prokaryotic organisms and viruses, as they can be amplified quickly, they are relatively easy to manipulate, and a large number of clones can be created. Preferred display packages include, for example, vegetative bacterial cells, bacterial spores, and most preferably, bacterial viruses (especially DNA viruses). However, the present invention also contemplates the use of eukaryotic cells, including yeast and their spores, as potential display packages.

In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating the variegated antibody display library of the present invention can be found in, for example, the Ladner et al. U.S. Patent No. 5,223,409; the Kang et al. International Publication No. WO 92/18619; the Dower et al. International Publication No. WO 91/17271; the Winter et al. International Publication WO 92/20791; the Markland et al. International Publication No. WO 92/15679; the Breitling et al. International Publication WO 93/01288; the McCafferty et al. International Publication No. WO 92/01047; the Garrad et al. International Publication No. WO 92/09690; the Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol. Biol.* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrard et al. (1991)

Bio/Technology 9:1373-1377; Hoogenboom et al. (1991) *Nuc. Acid Res.* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982. These systems can, with modifications described herein, be adapted for use in the subject method.

When the display is based on a bacterial cell, or a phage that is assembled periplasmically, the display means of the package will comprise at least two components. The first component is a secretion signal that directs the recombinant antibody to be localized on the extracellular side of the cell membrane (of the host cell when the display package is a phage). This secretion signal can be selected so as to be cleaved off by a signal peptidase to yield a processed, "mature" antibody. The second component is a display anchor protein that directs the display package to associate the test antibody with its outer surface. As described below, this anchor protein can be derived from a surface or coat protein native to the genetic package. When the display package is a bacterial spore, or a phage whose protein coating is assembled intracellularly, a secretion signal directing the antibody to the inner membrane of the host cell is unnecessary. In these cases, the means for arraying the variegated antibody library comprises a derivative of a spore or phage coat protein amenable for use as a fusion protein.

The antibody component of the display will comprise, at a minimum, one of either a V_H or V_L regions, e.g., cloned from B cells. It will be appreciated, however, that the V_H regions and/or the V_L regions may contain, in addition to the variable portion of the antibodies, all or a portion of the constant regions. Typically, the display library will include variable regions of both heavy and light chains in order to generate at least an Fv fragment. For clarity, embodiments described herein detail the minimal antibody display as comprising the use of cloned V_H regions to construct the fusion protein with the display anchor protein. However, it should be readily understood that similar embodiments are possible in which the role of the V_L and V_H chains are reversed in the construction of the display library. Where the display antibody is to include two or more chains, one chain can be provided as a

fusion protein with the genetic package, the other chain(s) can be secreted and become associated with the fusion protein.

Under certain circumstances, the V_H portion of the antibody display is derived from a library of different sequences, but the V_L chain is either absent or is a "fixed" V_L (i.e., the same V_L chain for every antibody of the display). Where, for example, the V_L portion of the display is fixed, the V_L chain can be contributed from a gene construct other than the construct encoding the V_H chain, or from the host cell itself (i.e., a light chain-producing myeloma cell), or added exogenously to the packages so as to recombine with V_H chains already displayed on their surface. However, it will generally be preferred that the V_L chain is derived from a variegated V_L library, e.g., also cloned from the same population of B cells from which the V_H gene is cloned, in which case a preferred embodiment places the V_L gene in the same construct as the V_H gene such that both may be readily recovered together.

When the desired antibody display is a multi-chain antibody (e.g., V_H and V_L are separate polypeptide chains), the cDNA encoding the light chain may be cloned directly into an appropriate site of the vector containing the heavy chain-coat protein library; or, alternatively, the light chain may be cloned as a separate library in a different plasmid vector, amplified, and subsequently the fragments cloned into the vector library encoding the heavy chain. In such circumstances, the V_L chain is cloned so that it is expressed with a signal peptide leader sequence that will direct its secretion into the periplasm of the host cell. For example, several leader sequences have been shown to direct the secretion of antibody sequences in *E. coli*, such as OmpA (Hsiung et al. *Bio/Technology* (1986) 4:991-995), and (Better et al. *Science* 240:1041-1043), phoA (Skerra and Pluckthun, *Science* (1988) 240:1038).
In the instance wherein the display package is a phage, the cloning site for the V_L chain sequences in the phagemid should be placed so that it does not substantially interfere with normal phage function. One such locus is the intergenic region as

described by Zinder and Boeke, (1982) *Gene* 19:1-10. In an illustrative embodiment comprising an M13 phage display library, the V_L sequence is preferably expressed at an equal or higher-level than the H_L-cpIII product (described below) to maintain a sufficiently high V_L concentration in the periplasm and provide efficient assembly (association) of V_L with V_H chains. For instance, a phagemid can be constructed to encode, as separate genes, both a V_H/coat fusion protein and a V_L chain. Under the appropriate induction, both chains are expressed and allowed to assemble in the periplasmic space of the host cell, the assembled antibody being linked to the phage particle by virtue of the V_H chain being a portion of a coat protein fusion construct. The number of possible combinations of heavy and light chains probably exceeds 10^{12}. To sample as many combinations as possible depends, in part, on the ability to recover large numbers of transformants. For phage with plasmid-like forms (as filamentous phage), electrotransformation provides an efficiency comparable to that of phage-transfection with *in vitro* packaging, in addition to a very high capacity for DNA input. This allows large amounts of vector DNA to be used to obtain very large numbers of transformants. The method described by Dower et al. (1988) *Nucleic Acids Res.*, 16:6127-6145, for example, may be used to transform fd-tet derived recombinants at the rate of about 10^7 transformants/ug of ligated vector into *E. coli* (such as strain MC1061), and libraries may be constructed in fd-tet Bl of up to about 3 x 10^8 members or more. Increasing DNA input and making modifications to the cloning protocol within the ability of the skilled artisan may produce increases of greater than about 10-fold in the recovery of transformants, providing libraries of up to 10^{10} or more recombinants.

In other embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined

by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity.

As will be apparent to those skilled in the art, in embodiments wherein high affinity antibodies are sought, an important criterion for the present selection method can be that it is able to discriminate between antibodies of different affinity for a particular antigen, and preferentially enrich for the antibodies of highest affinity. Applying the well known principles of antibody affinity and valence (i.e., avidity), it is understood that manipulating the display package to be rendered effectively monovalent can allow affinity enrichment to be carried out for generally higher binding affinities (i.e., binding constants in the range of 10^6 to 10^{10} M^{-1}) as compared to the broader range of affinities isolable using a multivalent display package. To generate the monovalent display, the natural (i.e., wild-type) form of the surface or coat protein used to anchor the antibody to the display can be added at a high enough level that it almost entirely eliminates inclusion of the antibody fusion protein in the display package. Thus, a vast majority of the display packages can be generated to include no more than one copy of the antibody fusion protein (see, for example, Garrad et al. (1991) *Bio/Technology* 9:1373-1377). In a preferred embodiment of a monovalent display library, the library of display packages will comprise no more than 5 to 10% polyvalent displays, and more preferably no more than 2% of the display will be polyvalent, and most preferably, no more than 1% polyvalent display packages in the population. The source of the wild-type anchor protein can be, for example, provided by a copy of the wild-type gene present on the same construct as the antibody fusion protein, or provided by a separate construct altogether. However, it will be equally clear that by similar manipulation, polyvalent displays can be generated to isolate a broader range of binding affinities. Such antibodies can be useful, for example, in purification protocols where avidity can be desirable.

In some instances it may be necessary to introduce an unstructured polypeptide linker region between portions of the chimeric protein, e.g., between the test antibody and display polypeptide. This linker can facilitate enhanced flexibility

of the chimeric protein allowing the test antibody to freely interact with a target by reducing steric hindrance between the two fragments, as well as allowing appropriate folding of each portion to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence $(Gly_4Ser)_3$ can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent Nos. 5,091,513 and 5,258,498. Naturally occurring unstructured linkers of human origin are preferred as they reduce the risk of immunogenicity.

In the instance wherein the display package is a phage, the cloning site for the test antibody gene sequences in the phagemid should be placed so that it does not substantially interfere with normal phage function. One such locus is the intergenic region as described by Zinder and Boeke, (1982) *Gene* 19:1-10.

i) Phages As Display Packages

Bacteriophage are attractive prokaryotic-related organisms for use in the subject method. Bacteriophage are excellent candidates for providing a display system of the variegated antibody library as there is little or no enzymatic activity associated with intact mature phage, and because their genes are inactive outside a bacterial host, rendering the mature phage particles metabolically inert. In general, the phage surface is a relatively simple structure. Phage can be grown easily in large numbers, they are amenable to the practical handling involved in many potential mass-screening programs, and they carry genetic information for their own synthesis within a small, simple package. As the antibody gene is inserted into the phage genome, choosing the appropriate phage to be employed in the subject method will generally depend primarily on whether (i) the genome of the phage allows introduction of the antibody gene either by tolerating additional genetic material or by having replaceable genetic material; (ii) the virion is capable of packaging the genome after accepting the insertion or substitution of genetic material; and (iii) the display of the antibody on the phage surface does not disrupt virion structure sufficiently to interfere with phage propagation.

One concern presented with the use of phage is that the morphogenetic pathway of the phage determines the environment in which the antibody will have opportunity to fold. Periplasmically assembled phage are preferred as the displayed antibodies may contain essential disulfides, and such antibodies may not fold correctly within a cell. However, in certain embodiments in which the display package forms intracellularly (e.g., where λ phage are used), it has been demonstrated in other instances that disulfide-containing antibodies can assume proper folding after the phage is released from the cell.

Another concern related to the use of phage, but also pertinent to the use of bacterial cells and spores as well, is that multiple infections could generate hybrid displays that carry the gene for one particular test antibody yet have two or more different test antibodies on their surfaces. Therefore, it can be preferable, though optional, to minimize this possibility by infecting cells with phage under conditions resulting in a low multiple-infection.

For a given bacteriophage, the preferred display means is a protein that is present on the phage surface (e.g., a coat protein). Filamentous phage can be described by a helical lattice; isometric phage, by an icosahedral lattice. Each monomer of each major coat protein sits on a lattice point and makes defined interactions with each of its neighbors. Proteins that fit into the lattice by making some, but not all, of the normal lattice contacts are likely to destabilize the virion by aborting formation of the virion as well as by leaving gaps in the virion so that the nucleic acid is not protected. Thus in bacteriophage, unlike the cases of bacteria and spores, it is generally important to retain in the antibody fusion proteins those residues of the coat protein that interact with other proteins in the virion. For example, when using the M13 cpVIII protein, the entire mature protein will generally be retained with the antibody fragment being added to the N-terminus of cpVIII, while on the other hand it can suffice to retain only the last 100 carboxy-terminal residues (or even fewer) of the M13 cpIII coat protein in the antibody fusion protein.

Under the appropriate induction, the test antibody library is expressed and exported, as part of the fusion protein, to the bacterial cytoplasm, such as when the λ phage is employed. The induction of the fusion protein(s) may be delayed until some replication of the phage genome, synthesis of some of the phage structural-proteins, and assembly of some phage particles has occurred. The assembled protein chains then interact with the phage particles via the binding of the anchor protein on the outer surface of the phage particle. The cells are lysed and the phage bearing the library-encoded test antibody (that corresponds to the specific library sequences carried in the DNA of that phage) are released and isolated from the bacterial debris. To enrich for and isolate phage which encodes a selected test antibody, and thus to ultimately isolate the nucleic acid sequences (the antibody gene) themselves, phage harvested from the bacterial debris are affinity-purified. As described below, when a test antibody which specifically binds a particular target is desired, the target can be used to retrieve phage displaying the desired test antibody. The phage so obtained may then be amplified by infecting into host cells. Additional rounds of affinity enrichment followed by amplification may be employed until the desired level of enrichment is reached.

The enriched antibody-phage can also be screened with additional detection-techniques such as expression plaque (or colony) lift (see, e.g., Young and Davis, *Science* (1983) 222:778-782) whereby a labeled target is used as a probe.

a) Filamentous Phage

Filamentous bacteriophages, which include M13, fl, fd, Ifl, Ike, Xf, Pfl, and Pf3, are a group of related viruses that infect bacteria. They are termed filamentous because they are long, thin particles comprised of an elongated capsule that envelopes the deoxyribonucleic acid (DNA) that forms the bacteriophage genome. The F pili filamentous bacteriophage (Ff phage) infect only gram-negative bacteria by specifically adsorbing to the tip of F pili, and include fd, fl and M13.

Compared to other bacteriophage, filamentous phage in general are attractive and M13 in particular is especially attractive because: (i) the 3-D structure of the virion is known; (ii) the processing of the coat protein is well understood; (iii) the

genome is expandable; (iv) the genome is small; (v) the sequence of the genome is known; (vi) the virion is physically resistant to shear, heat, cold, urea, guanidinium chloride, low pH, and high salt; (vii) the phage is a sequencing vector so that sequencing is especially easy; (viii) antibiotic-resistance genes have been cloned into the genome with predictable results (Hines et al. (1980) *Gene* 11:207-218); (ix) it is easily cultured and stored, with no unusual or expensive media requirements for the infected cells, (x) it has a high burst size, each infected cell yielding 100 to 1000 M13 progeny after infection; and (xi) it is easily harvested and concentrated (Salivar et al. (1964) *Virology* 24: 359-371). The entire life cycle of the filamentous phage M13, a common cloning and sequencing vector, is well understood. The genetic structure of M13 is well known, including the complete sequence (Schaller et al. in *The Single-Stranded DNA Phages* eds. Denhardt et al. (NY: CSHL Press, 1978)), the identity and function of the ten genes, and the order of transcription and location of the promoters, as well as the physical structure of the virion (Smith et al. (1985) *Science* 228:1315-1317; Raschad et al. (1986) *Microbiol Dev* 50:401-427; Kuhn et al. (1987) *Science* 238:1413-1415; Zimmerman et al. (1982) *J Biol Chem* 257:6529-6536; and Banner et al. (1981) *Nature* 289:814-816). Because the genome is small (6423 bp), cassette mutagenesis is practical on RF M13 (*Current Protocols in Molecular Biology,* eds. Ausubel et al. (NY: John Wiley & Sons, 1991)), as is single-stranded oligonucleotide directed mutagenesis (Fritz et al. in *DNA Cloning,* ed by Glover (Oxford, UK: IRC Press, 1985)). M13 is a plasmid and transformation system in itself, and an ideal sequencing vector. M13 can be grown on Rec- strains of *E. coli.* The M13 genome is expandable (Messing et al. in *The Single-Stranded DNA Phages*, eds Denhardt et al. (NY: CSHL Press, 1978) pages 449-453; and Fritz et al., *supra*) and M13 does not lyse cells. Extra genes can be inserted into M13 and will be maintained in the viral genome in a stable manner.

The mature capsule or Ff phage is comprised of a coat of five phage-encoded gene products: cpVIII, the major coat protein product of gene VIII that forms the bulk of the capsule; and four minor coat proteins, cpIII and cpIV at one end of the capsule and cpVII and cpIX at the other end of the capsule. The length of the

capsule is formed by 2500 to 3000 copies of cpVIII in an ordered helix array that forms the characteristic filament structure. The gene III-encoded protein (cpIII) is typically present in 4 to 6 copies at one end of the capsule and serves as the receptor for binding of the phage to its bacterial host in the initial phase of infection. For detailed reviews of Ff phage structure, see Rasched et al., *Microbiol. Rev.*, 50:401-427 (1986); and Model et al., in *The Bacteriophages, Volume 2*, R. Calendar, Ed., Plenum Press, pp. 375-456 (1988).

The phage particle assembly involves extrusion of the viral genome through the host cell's membrane. Prior to extrusion, the major coat protein cpVIII and the minor coat protein cpIII are synthesized and transported to the host cell's membrane. Both cpVIII and cpIII are anchored in the host cell membrane prior to their incorporation into the mature particle. In addition, the viral genome is produced and coated with cpV protein. During the extrusion process, cpV-coated genomic DNA is stripped of the cpV coat and simultaneously recoated with the mature coat proteins. Both cpIII and cpVIII proteins include two domains that provide signals for assembly of the mature phage particle. The first domain is a secretion signal that directs the newly synthesized protein to the host cell membrane. The secretion signal is located at the amino terminus of the polypeptide and targets the polypeptide at least to the cell membrane. The second domain is a membrane anchor domain that provides signals for association with the host cell membrane and for association with the phage particle during assembly. This second signal for both cpVIII and cpIII comprises at least a hydrophobic region for spanning the membrane.

The 50-amino acid mature gene VIII coat protein (cpVIII) is synthesized as a 73 amino acid precoat (Ito et al. (1979) *PNAS* 76:1199-1203). cpVIII has been extensively studied as a model membrane protein because it can integrate into lipid bilayers such as the cell membrane in an asymmetric orientation with the acidic amino terminus toward the outside and the basic carboxy terminus toward the inside of the membrane. The first 23 amino acids constitute a typical signal-sequence that causes the nascent polypeptide to be inserted into the inner cell membrane. An *E. coli* signal peptidase (SP-I) recognizes amino acids 18, 21, and 23, and, to a lesser

extent, residue 22, and cuts between residues 23 and 24 of the precoat (Kuhn et al. (1985) *J. Biol. Chem.* 260:15914-15918; and Kuhn et al. (1985) *J. Biol. Chem.* 260:15907-15913). After removal of the signal sequence, the amino terminus of the mature coat is located on the periplasmic side of the inner membrane; the carboxy terminus is on the cytoplasmic side. About 3000 copies of the mature coat protein associate side-by-side in the inner membrane.

The sequence of gene VIII is known, and the amino acid sequence can be encoded on a synthetic gene. Mature gene VIII protein makes up the sheath around the circular ssDNA. The gene VIII protein can be a suitable anchor protein because its location and orientation in the virion are known (Banner et al. (1981) *Nature* 289:814-816). Preferably, the antibody is attached to the amino terminus of the mature M13 coat protein to generate the phage display library. As set out above, manipulation of the concentration of both the wild-type cpVIII and Ab/cpVIII fusion in an infected cell can be utilized to decrease the avidity of the display and thereby enhance the detection of high affinity antibodies directed to the target(s).
Another vehicle for displaying the antibody is by expressing it as a domain of a chimeric gene containing part or all of gene III, e.g., encoding cpIII. When monovalent displays are required, expressing the antibody as a fusion protein with cpIII can be a preferred embodiment, as manipulation of the ratio of wild-type cpIII to chimeric cpIII during formation of the phage particles can be readily controlled. This gene encodes one of the minor coat proteins of M13. Genes VI, VII, and IX also encode minor coat proteins. Each of these minor proteins is present in about 5 copies per virion and is related to morphogenesis or infection. In contrast, the major coat protein is present in more than 2500 copies per virion. The gene VI, VII, and IX proteins are present at the ends of the virion; these three proteins are not posttranslationally processed (Rasched et al. (1986) *Ann Rev. Microbiol.* 41:507-541). In particular, the single-stranded circular phage DNA associates with about five copies of the gene III protein and is then extruded through the patch of membrane-associated coat protein in such a way that the DNA is encased in a helical

sheath of protein (Webster et al. in *The Single-Stranded DNA Phages,* eds. Dressler et al. (NY:CSHL Press, 1978).

Manipulation of the sequence of cpIII has demonstrated that the C-terminal 23-amino acid residue stretch of hydrophobic amino acids normally responsible for a membrane anchor function can be altered in a variety of ways and retain the capacity to associate with membranes. Ff phage-based expression vectors were first described in which the cpIII amino acid residue sequence was modified by insertion of polypeptide "targets" (Parmely et al., *Gene* (1988) 73:305-318; and Cwirla et al., *PNAS* (1990) 87:6378-6382) or an amino acid residue sequence defining a single chain antibody domain (McCafferty et al., *Science* (1990) 348:552-554). It has been demonstrated that insertions into gene III can result in the production of novel protein domains on the virion outer surface. (Smith (1985) *Science* 228:1315-1317; and de la Cruz et al. (1988) *J. Biol. Chem.* 263:4318-4322). The antibody gene may be fused to gene III at the site used by Smith and by de la Cruz et al., at a codon corresponding to another domain boundary or to a surface loop of the protein, or to the amino terminus of the mature protein.

Generally, the successful cloning strategy utilizing a phage coat protein, such as cpIII of filamentous phage fd, will provide expression of an antibody chain fused to the N-terminus of a coat protein (e.g., cpIII) and transport to the inner membrane of the host where the hydrophobic domain in the C-terminal region of the coat protein anchors the fusion protein in the membrane, with the N-terminus containing the antibody chain protruding into the periplasmic space.

Similar constructions could be made with other filamentous phage. Pf3 is a well known filamentous phage that infects *Pseudomonos aerugenosa* cells that harbor an IncP-I plasmid. The entire genome has been sequenced ((Luiten et al. (1985) *J. Virol.* 56:268-276) and the genetic signals involved in replication and assembly are known (Luiten et al. (1987) DNA 6:129-137). The major coat protein of PF3 is unusual in having no signal antibody to direct its secretion. The sequence has charged residues ASP-7, ARG-37, LYS-40, and PHE44 which is consistent with the amino terminus being exposed. Thus, to cause an antibody to appear on the

surface of Pf3, a tripartite gene can be constructed which comprises a signal sequence known to cause secretion in *P. aerugenosa*, fused in-frame to a gene fragment encoding the antibody sequence, which is fused in-frame to DNA encoding the mature Pf3 coat protein. Optionally, DNA encoding a flexible linker of one to 10 amino acids is introduced between the antibody gene fragment and the Pf3 coat-protein gene. This tripartite gene is introduced into Pf3 so that it does not interfere with expression of any Pf3 genes. Once the signal sequence is cleaved off, the antibody is in the periplasm and the mature coat protein acts as an anchor and phage-assembly signal.

b) Bacteriophage ϕX174

The bacteriophage ϕX174 is a very small icosahedral virus that has been thoroughly studied by genetics, biochemistry, and electron microscopy (see *The Single Stranded DNA Phages* (eds. Den hardt et al. (NY:CSHL Press, 1978)). Three gene products of ϕX174 are present on the outside of the mature virion: F (capsid), G (major spike protein, 60 copies per virion), and H (minor spike protein, 12 copies per virion). The G protein comprises 175 amino acids, while H comprises 328 amino acids. The F protein interacts with the single-stranded DNA of the virus. The proteins F, G, and H are translated from a single mRNA in the viral infected cells. As the virus is so tightly constrained because several of its genes overlap, ϕX174 is not typically used as a cloning vector due to the fact that it can accept very little additional DNA. However, mutations in the viral G gene (encoding the G protein) can be rescued by a copy of the wild-type G gene carried on a plasmid that is expressed in the same host cell (Chambers et al. (1982) *Nuc. Acid Res.* 10:6465-6473). In one embodiment, one or more stop codons are introduced into the G gene so that no G protein is produced from the viral genome. The variegated antibody gene library can then be fused with the nucleic acid sequence of the H gene. An amount of the viral G gene equal to the size of antibody gene fragment is eliminated from the ϕX174 genome, such that the size of the genome is ultimately unchanged. Thus, in host cells also transformed with a second plasmid expressing the wild-type G protein, the production of viral particles from the mutant virus is rescued by the

exogenous G protein source. Where it is desirable that only one test antibody be displayed per ϕX174 particle, the second plasmid can further include one or more copies of the wild-type H protein gene so that a mix of H and test antibody/H proteins will be predominated by the wild-type H upon incorporation into phage particles.

c) Large DNA Phage

Phage such as λ or T4 have much larger genomes than do M13 or ϕX174, and have more complicated 3-D capsid structures than Ml3 or ϕX174, with more coat proteins to choose from. In embodiments of the invention whereby the test antibody library is processed and assembled into a functional form and associates with the bacteriophage particles within the cytoplasm of the host cell, bacteriophage λ and derivatives thereof are examples of suitable vectors. The intracellular morphogenesis of phage λ can potentially prevent protein domains that ordinarily contain disulfide bonds from folding correctly. However, variegated libraries expressing a population of functional antibodies, which include such bonds, have been generated in λ phage. (Huse et al. (1989) *Science* 246:1275-1281; Mullinax et al. (1990) *PNAS* 87:8095-8099; and Pearson et al. (1991) *PNAS* 88:2432-2436). Such strategies take advantage of the rapid construction and efficient transformation abilities of λ phage.

When used for expression of antibody sequences (exogenous nucleotide sequences), may be readily inserted into a λ vector. For instance, variegated antibody libraries can be constructed by modification of λ ZAP II through use of the multiple cloning site of a λ ZAP II vector (Huse et al. *supra*).

ii) Bacterial Cells as Display Packages

Recombinant antibodies are able to cross bacterial membranes after the addition of appropriate secretion signal sequences to the N-terminus of the protein (Better et al (1988) *Science* 240:1041-1043; and Skerra et al. (1988) *Science* 240:1038-1041). In addition, recombinant antibodies have been fused to outer membrane proteins for surface presentation. For example, one strategy for displaying antibodies on bacterial cells comprises generating a fusion protein by

inserting the antibody into cell surface exposed portions of an integral outer membrane protein (Fuchs et al. (1991) *Bio/Technology* 9:1370-1372). In selecting a bacterial cell to serve as the display package, any well-characterized bacterial strain will typically be suitable, provided the bacteria may be grown in culture, engineered to display the test antibody library on its surface, and is compatible with the particular affinity selection process practiced in the subject method. Among bacterial cells, the preferred display systems include *Salmonella typhirnurium, Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae, Klebsiella pneumonia, Neisseria gonorrhoeae, Neisseria meningitidis, Bacteroides nodosus, Moraxella bovis,* and especially *Escherichia coli*. Many bacterial cell surface proteins useful in the present invention have been characterized, and works on the localization of these proteins and the methods of determining their structure include Benz et al. (1988) *Ann Rev Microbiol* 42: 359-393; Balduyck et al. (1985) *Biol Chem Hoppe-Seyler* 366:9-14; Ehrmann et al (1990) *PNAS* 87:7574-7578; Heijne et al. (1990) *Protein Engineering* 4:109-112; Ladner et al. U.S. Patent No. 5,223,409; Ladner et al. WO 88/06630; Fuchs et al. (1991) *Bio/technology* 9:1370-1372; and Goward et al. (1992) *TIBS* 18:136-140.

To further illustrate, the LamB protein of *E coli* is a well understood surface protein that can be used to generate a variegated library of test antibodies on the surface of a bacterial cell (see, for example, Ronco et al. (1990) *Biochemie* 72:183-189; van der Weit et al. (1990) *Vaccine* 8:269-277; Charabit et al. (1988) *Gene* 70:181-189; and Ladner U.S. Patent No. 5,222,409). LamB of *E. coli* is a porin for maltose and maltodextrin transport, and serves as the receptor for adsorption of bacteriophages λ and K10. LamB is transported to the outer membrane if a functional N-terminal signal sequence is present (Benson et al. (1984) *PNAS* 81:3830-3834). As with other cell surface proteins, LamB is synthesized with a typical signal-sequence that is subsequently removed. Thus, the variegated antibody gene library can be cloned into the LamB gene such that the resulting library of fusion proteins comprise a portion of LamB sufficient to anchor the protein to the cell membrane with the test antibody fragment oriented on the extracellular side of

the membrane. Secretion of the extracellular portion of the fusion protein can be facilitated by inclusion of the LamB signal sequence, or other suitable signal sequence, as the N-terminus of the protein.

The *E. coli* LamB has also been expressed in functional form in *S. typhimurium* (Harkki et al. (1987) *Mol Gen Genet* 209:607-611), *V. cholerae* (Harkki et al. (1986) *Microb Pathol* 1:283-288), and *K. pneumonia* (Wehmeier et al. (1989) *Mol Gen Genet* 215:529-536), so that one could display a population of test antibodies in any of these species as a fusion to *E. coli* LamB. Moreover, *K. pneumonia* expresses a maltoporin similar to LamB which could also be used. In *P. aeruginosa*, the Dl protein (a homologue of LamB) can be used (Trias et al. (1988) *Biochem Biophys Acta* 938:493-496). Similarly, other bacterial surface proteins, such as PAL, OmpA, OmpC, OmpF, PhoE, pilin, BtuB, FepA, FhuA, IutA, FecA and FhuE, may be used in place of LamB as a portion of the display means in a bacterial cell.

In another exemplary embodiment, the fusion protein can be derived using the FliTrx™ Random Antibody Display Library (Invitrogen). That library is a diverse population of random dodecaantibodies inserted within the thioredoxin active-site loop inside the dispensable region of the bacterial flagellin gene (fliC). The resultant recombinant fusion protein (FLITRX) is exported and assembled into partially functional flagella on the bacterial cell surface, displaying the random antibody library.

Antibodies are fused in the middle of thioredoxin, therefore, both their N- and C-termini are anchored by thioredoxin's tertiary structure. This results in the display of a constrained antibody. By contrast, phage display proteins are fused to the N-terminus of phage coat proteins in an unconstrained manner. The unconstrained molecules possess many degrees of conformational freedom that may result in the lack of proper interaction with the target molecule. Without proper interaction, many potential protein-protein interactions may be missed. Moreover, phage display is limited by the low expression levels of bacteriophage coat proteins. FliTrx™ and similar methods can overcome this limitation by using a

strong promoter to drive expression of the test antibody fusions that are displayed as multiple copies.

According to the present invention, it is contemplated that the FliTrx vector can be modified to provide, similar to the illustrated vectors of the attached figures, a vector which is differentially spliced in mammalian cells to yield a secreted, soluble test antibody.

iii) Bacterial Spores as Display Packages

Bacterial spores also have desirable properties as display package candidates in the subject method. For example, spores are much more resistant than vegetative bacterial cells or phage to chemical and physical agents, and hence permit the use of a great variety of affinity selection conditions. Also, Bacillus spores neither actively metabolize nor alter the proteins on their surface. However, spores have the disadvantage that the molecular mechanisms that trigger sporulation are less well worked out than is the formation of M13 or the export of protein to the outer membrane of *E. coli*, though such a limitation is not a serious detractant from their use in the present invention.

Bacteria of the genus Bacillus form endospores which are extremely resistant to damage by heat, radiation, desiccation, and toxic chemicals (reviewed by Losick et al. (1986) *Ann Rev Genet* 20:625-669). This phenomenon is attributed to extensive intermolecular cross-linking of the coat proteins. In certain embodiments of the subject method, such as those that include relatively harsh affinity separation steps, Bacillus spores can be the preferred display package. Endospores from the genus Bacillus are more stable than are, for example, exospores from Streptomyces. Moreover, *Bacillus subtilis* forms spores in 4 to 6 hours, whereas Streptomyces species may require days or weeks to sporulate. In addition, genetic knowledge and manipulation is much more developed for *B. subtilis* than for other spore-forming bacteria.

Viable spores that differ only slightly from wild-type are produced in *B. subtilis* even if any one of four coat proteins is missing (Donovan et al. (1987) *J Mol Biol* 196:1-10). Moreover, plasmid DNA is commonly included in spores, and

plasmid encoded proteins have been observed on the surface of Bacillus spores (Debro et al. (1986) *J Bacteriol* 165:258-268). Thus, it can be possible during sporulation to express a gene encoding a chimeric coat protein comprising an antibody of the variegated gene library, without interfering materially with spore formation.

To illustrate, several polypeptide components of *B. subtilis* spore coat (Donovan et al. (1987) *J Mol Biol* 196:1-10) have been characterized. The sequences of two complete coat proteins and amino-terminal fragments of two others have been determined. Fusion of the test antibody sequence to cotC or cotD fragments is likely to cause the antibody to appear on the spore surface. The genes of each of these spore coat proteins are preferred as neither cotC or cotD are post-translationally modified (see Ladner et al. U.S. Patent No. 5,223,409).

iv) Selecting Antibodies from the Display Mode

Upon expression, the variegated antibody display is subjected to affinity enrichment in order to select for test antibodies that bind preselected targets. The term "affinity separation" or "affinity enrichment" includes, but is not limited to: (1) affinity chromatography utilizing immobilized targets, (2) immunoprecipitation using soluble targets, (3) fluorescence activated cell sorting, (4) agglutination, and (5) plaque lifts. In each embodiment, the library of display packages is ultimately separated based on the ability of the associated test antibody to bind the target of interest. See, for example, the Ladner et al. U.S. Patent No. 5,223,409; the Kang et al. International Publication No. WO 92/18619; the Dower et al. International Publication No. WO 91/17271; the Winter et al. International Publication WO 92/20791; the Markland et al. International Publication No. WO 92/15679; the Breitling et al. International Publication WO 93/01288; the McCafferty et al. International Publication No. WO 92/01047; the Garrard et al. International Publication No. WO 92/09690; and the Ladner et al. International Publication No. WO 90/02809. In most preferred embodiments, the display library will be pre-enriched for antibodies specific for the target by first contacting the display library with any negative controls or other targets for which differential binding by the test

antibody is desired. Subsequently, the non-binding fraction from that pre-treatment step is contacted with the target and antibodies from the display which are able to specifically bind the target are isolated.

With respect to affinity chromatography, it will be generally understood by those skilled in the art that a great number of chromatography techniques can be adapted for use in the present invention, ranging from column chromatography to batch elution, and including ELISA and biopanning techniques. Typically, where the target is a component of a cell, rather than a whole cell, the target is immobilized on an insoluble carrier, such as sepharose or polyacrylamide beads, or, alternatively, the wells of a microtitre plate. As described below, in instances where no purified source of the target is readily available, such as the case with many cell surface receptors, the cells on which the target is displayed may serve as the insoluble matrix carrier.

The population of display packages is applied to the affinity matrix under conditions compatible with the binding of the test antibody to a target. The population is then fractionated by washing with a solute that does not greatly effect specific binding of antibodies to the target, but which substantially disrupts any non-specific binding of the display package to the target or matrix. A certain degree of control can be exerted over the binding characteristics of the antibodies recovered from the display library by adjusting the conditions of the binding incubation and subsequent washing. The temperature, pH, ionic strength, divalent cation concentration, and the volume and duration of the washing can select for antibodies within a particular range of affinity and specificity. Selection based on slow dissociation rate, which is usually predictive of high affinity, is a very practical route. This may be done either by continued incubation in the presence of a saturating amount of free hapten (if available), or by increasing the volume, number, and length of the washes. In each case, the rebinding of dissociated antibody-display package is prevented, and with increasing time, antibody-display packages of higher and higher affinity are recovered. Moreover, additional modifications of the binding and washing procedures may be applied to find antibodies with special

characteristics. The affinities of some antibodies are dependent on ionic strength or cation concentration. This is a useful characteristic for antibodies to be used in affinity purification of various proteins when gentle conditions for removing the protein from the antibody are required. Specific examples are antibodies which depend on Ca^{++} for binding activity and which lose or gain binding affinity in the presence of EGTA or other metal chelating agent. Such antibodies may be identified in the recombinant antibody library by a double screening technique isolating first those that bind the target in the presence of Ca^{++}, and by subsequently identifying those in this group that fail to bind in the presence of EGTA.

After "washing" to remove non-specifically bound display packages, when desired, specifically bound display packages can be eluted by either specific desorption (using excess target) or non-specific desorption (using pH, polarity reducing agents, or chaotropic agents). In preferred embodiments, the elution protocol does not kill the organism used as the display package such that the enriched population of display packages can be further amplified by reproduction. The list of potential eluants includes salts (such as those in which one of the counter ions is Na^+, NH_4^+, Rb^+, SO_4^{2-}, $H_2PO_4^-$, citrate, K^+, Li^+, Cs^+, HSO_4^-, CO_3^{2-}, Ca^{2+}, Sr^{2+}, Cl^-, PO_4^{2-}, HCO_3^-, Mg_2^+, Ba_2^+, Br^-, HPO_4^{2-}, or acetate), acid, heat, and, when available, soluble forms of the target target (or analogs thereof). Because bacteria continue to metabolize during the affinity separation step and are generally more susceptible to damage by harsh conditions, the choice of buffer components (especially eluates) can be more restricted when the display package is a bacteria rather than for phage or spores. Neutral solutes, such as ethanol, acetone, ether, or urea, are examples of other agents useful for eluting the bound display packages. In preferred embodiments, affinity enriched display packages are iteratively amplified and subjected to further rounds of affinity separation until enrichment of the desired binding activity is detected. In certain embodiments, the specifically bound display packages, especially bacterial cells, need not be eluted per se, but

rather, the matrix-bound display packages can be used directly to inoculate a suitable growth media for amplification.

Where the display package is a phage particle, the fusion protein generated with the coat protein can interfere substantially with the subsequent amplification of eluted phage particles, particularly in embodiments wherein the cpIII protein is used as the display anchor. Even though present in only one of the 5-6 tail fibers, some antibody constructs because of their size and/or sequence, may cause severe defects in the infectivity of their carrier phage. This causes a loss of phage from the population during reinfection and amplification following each cycle of panning. In one embodiment, the antibody can be derived on the surface of the display package so as to be susceptible to proteolytic cleavage that severs the covalent linkage of at least the target binding sites of the displayed antibody from the remaining package. For instance, where the cpIII coat protein of M13 is employed, such a strategy can be used to obtain infectious phage by treatment with an enzyme that cleaves between the test antibody portion and cpIII portion of a tail fiber fusion protein (e.g., by using an enterokinase cleavage recognition sequence).

To further minimize problems associated with defective infectivity, DNA prepared from the eluted phage can be transformed into host cells by electroporation or well known chemical means. The cells are cultivated for a period of time sufficient for marker expression, and selection is applied as typically done for DNA transformation. The colonies are amplified, and phage harvested for a subsequent round(s) of panning.

After isolation of display packages that encode antibodies having a desired binding specificity for the target, the test antibodies for each of the purified display packages can be tested for biological activity in the secretion mode of the subject method.

B. Secretion Mode

In the "secretion mode," the combinatorial antibody library, which has been enriched in the display mode, is transfected into and expressed by eukaryotic cells.

In this mode, the test antibodies are secreted by the host cells and screened for biological activity.

In preferred embodiments, as illustrated in the drawings, the subject vectors are constructed to include eukaryotic splice sites such that, in the mature mRNA, elements required for the display mode in prokaryotic cells are spliced out – at least those elements which would interfere with the secretion mode. A variety of naturally and non-naturally occurring splice sites are available in the art and can be selected for, e.g., optimization in particular eukaryotic cells selected.

In preferred embodiments, the vectors of the subject invention are used to transfect a cell that can be co-cultured with a target cell. A biologically active protein secreted by the cells expressing the combinatorial library will diffuse to neighboring target cells and induce a particular biological response, such as to illustrate, proliferation or differentiation, or activation of a signal transduction pathway which is directly detected by other phenotypic criteria. The pattern of detection of biological activity will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing antibodies having certain activity in the assay. Likewise, antagonists of a given factor can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells from the effect of exogenous factor added to the culture media.

To further illustrate, target cells are cultured in 24-well microtitre plates. Other cells are transfected with the combinatorial antibody library, recovered after the display mode step, and cultured in cell culture inserts (e.g., Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant test antibodies secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for a secreted test antibody to produce a measurable response in the target cells, the inserts are removed and the effect of the antibodies on the target cells determined. For example, where the target cell is a neural crest

cell and the activity desired from the test antibodies is the induction of neuronal differentiation, then fluorescently labeled antibodies specific for Islet-1 or other neuronal markers can be used to score for induction in the target cells as indicative of a functional neurotrophic antibody in that well. Cells from the inserts corresponding to wells that score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active antibody is identified. When screening for bioactivity of test antibodies, intracellular second messenger generation can be measured directly. For instance, a variety of intracellular effectors have been identified as being receptor- or ion channel-regulated, including adenylyl cyclase, cyclic GMP, phosphodiesterases, phosphoinositidases, phosphoinositol kinases, and phospholipases, as well as a variety of ions.

In one embodiment, the GTPase enzymatic activity by G proteins can be measured in plasma membrane preparations by determining the breakdown of $\alpha^{32}P$ GTP using techniques that are known in the art (For example, see *Signal Transduction: A Practical Approach*. G. Milligan, Ed. Oxford University Press, Oxford England). When receptors that modulate cAMP are tested, it will be possible to use standard techniques for cAMP detection, such as competitive assays which quantitate [^{3}H]cAMP in the presence of unlabelled cAMP.

Certain receptors and ion channels stimulate the activity of phospholipase C which stimulates the breakdown of phosphatidylinositol 4,5, bisphosphate to 1,4,5-IP3 (which mobilizes intracellular Ca++) and diacylglycerol (DAG) (which activates protein kinase C). Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. DAG can also be measured using thin-layer chromatography. Water-soluble derivatives of all three inositol lipids (IP1, IP2, IP3) can also be quantitated using radiolabelling techniques or HPLC.

The other product of PIP2 breakdown, DAG can also be produced from phosphatidyl choline. The breakdown of this phospholipid in response to receptor-mediated signaling can also be measured using a variety of radiolabelling techniques.

The activation of phospholipase A2 can easily be quantitated using known techniques, including, for example, the generation of arachadonate in the cell. In various cells, e.g., mammalian cells, specific proteases are induced or activated in each of several arms of divergent signaling pathways. These may be independently monitored by following their unique activities with substrates specific for each protease.

In the case of certain receptors and ion channels, it may be desirable to screen for changes in cellular phosphorylation. Such assay formats may be useful when, for example, the assay is designed to detect an agonist or antagonist of a receptor kinase or phosphatase. For example, immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using anti-phosphotyrosine, anti-phosphoserine, or anti-phosphothreonine antibodies. In addition, tests for phosphorylation could be also useful when the receptor itself may not be a kinase, but activates protein kinases or phosphatase that function downstream in the signal transduction pathway.

One such cascade is the MAP kinase pathway that appears to mediate mitogenic, differentiation, and stress responses in different cell types. Stimulation of growth factor receptors results in Ras activation followed by the sequential activation of c-Raf, MEK, and p44 and p42 MAP kinases (ERK1 and ERK2). Activated MAP kinase then phosphorylates many key regulatory proteins, including p90RSK and Elk-1 that are phosphorylated when MAP kinase translocates to the nucleus. Homologous pathways exist in mammalian and yeast cells. For instance, an essential part of the *S. cerevisiae* pheromone signaling pathway is comprised of a protein kinase cascade composed of the products of the STE11, STE7, and FUS3/KSS1 genes (the latter pair are distinct and functionally redundant). Accordingly, phosphorylation and/or activation of members of this kinase cascade can be detected and used to quantitate receptor engagement. Phosphotyrosine specific antibodies are available to measure increases in tyrosine phosphorylation and phospho-specific antibodies are commercially available (New England Biolabs, Beverly, MA).

In yet another embodiment, the signal transduction pathway of interest may upregulate expression or otherwise activate an enzyme which is capable of modifying a substrate which can be added to the cell. The signal can be detected by using a detectable substrate, in which case lose of the substrate signal is monitored, or alternatively, by using a substrate which produces a detectable product. In preferred embodiments, the conversion of the substrate to product by the activated enzyme produces a detectable change in optical characteristics of the test cell, e.g., the substrate and/or product is chromogenically or fluorogenically active. In an illustrative embodiment the signal transduction pathway causes a change in the activity of a proteolytic enzyme, altering the rate at which it cleaves a substrate peptide (or simply activates the enzyme towards the substrate). The peptide includes a fluorogenic donor radical, e.g., a fluorescence emitting radical, and an acceptor radical, e.g., an aromatic radical that absorbs the fluorescence energy of the fluorogenic donor radical when the acceptor radical and the fluorogenic donor radical are covalently held in close proximity. See, for example, USSN 5,527,681, 5,506,115, 5,429,766, 5,424,186, and 5,316,691; and Capobianco et al. (1992) Anal Biochem 204:96-102. For example, the substrate peptide has a fluorescence donor group such as 1-aminobenzoic acid (anthranilic acid or ABZ) or aminomethylcoumarin (AMC) located at one position on the peptide and a fluorescence quencher group, such as lucifer yellow, methyl red or nitrobenzo-2-oxo-1,3-diazole (NBD), at a different position near the distal end of the peptide. A cleavage site for the activated enzyme will be disposed between each of the sites for the donor and acceptor groups. The intramolecular resonance energy transfer from the fluorescence donor molecule to the quencher will quench the fluorescence of the donor molecule when the two are sufficiently proximate in space, e.g., when the peptide is intact. Upon cleavage of the peptide, however, the quencher is separated from the donor group, leaving behind a fluorescent fragment. Thus, activation of the enzyme results in cleavage of the detection peptide, and dequenching of the fluorescent group.

In still other embodiments, the detectable signal can be produced by use of enzymes or chromogenic/fluorscent probes whose activities are dependent on the concentration of a second messenger, e.g., such as calcium, hydrolysis products of inositol phosphate, cAMP, etc. For example, the mobilization of intracellular calcium or the influx of calcium from outside the cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca++-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) Environ Health Perspect 84:45-56). As an exemplary method of Ca++ detection, cells could be loaded with the Ca++sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca++ measured using a fluorometer.

As certain embodiments described above suggest, in addition to directly measuring second messenger production, the signal transduction activity of a receptor or ion channel pathway can be measured by detection of a transcription product, e.g., by detecting receptor/channel-mediated transcriptional activation (or repression) of a gene(s). Detection of the transcription product includes detecting the gene transcript, detecting the product directly (e.g., by immunoassay) or detecting an activity of the protein (e.g., such as an enzymatic activity or chromogenic/fluorogenic activity); each of which is generally referred to herein as a means for detecting expression of the indicator gene. The indicator gene may be an unmodified endogenous gene of the host cell, a modified endogenous gene, or a part of a completely heterologous construct, e.g., as part of a reporter gene construct.

In one embodiment, the indicator gene is an unmodified endogenous gene. For example, the instant method can rely on detecting the transcriptional level of such endogenous genes as the c-fos gene (e.g., in mammalian cells) or the Bar1 or Fus1 genes (e.g., in yeast cells) in response to such signal transduction pathways as originating from G protein coupled receptors.

In certain instances, it may be desirable to increase the level of transcriptional activation of the endogenous indicator gene by the signal pathway in order to, for example, improve the signal-to-noise of the test system, or to adjust the

level of response to a level suitable for a particular detection technique. In one embodiment, the transcriptional activation ability of the signal pathway can be amplified by the overexpression of one or more of the proteins involved in the intracellular signal cascade, particularly enzymes involved in the pathway. For example, increased expression of Jun kinases (JNKs) can potentiate the level of transcriptional activation by a signal in an MEKK/JNKK pathway. Likewise, overexpression of one or more signal transduction proteins in the yeast pheromone pathway can increase the level of Fus1 and/or Bar1 expression. This approach can, of course, also be used to potentiate the level of transcription of a heterologous reporter gene as well.

In other embodiments, the sensitivity of an endogenous indicator gene can be enhanced by manipulating the promoter sequence at the natural locus for the indicator gene. Such manipulation may range from point mutations to the endogenous regulatory elements to gross replacement of all or substantial portions of the regulatory elements. In general, manipulation of the genomic sequence for the indicator gene can be carried out using techniques known in the art, including homologous recombination.

In another exemplary embodiment, the promoter (or other transcriptional regulatory sequences) of the endogenous gene can be "switched out" with a heterologous promoter sequence, e.g., to form a chimeric gene at the indicator gene locus. Again, using such techniques as homologous recombination, the regulatory sequence can be so altered at the genomic locus of the indicator gene.

In still another embodiment, a heterologous reporter gene construct can be used to provide the function of an indicator gene. Reporter gene constructs are prepared by operatively linking a reporter gene with at least one transcriptional regulatory element. If only one transcriptional regulatory element is included it must be a regulatable promoter. At least one the selected transcriptional regulatory elements must be indirectly or directly regulated by the activity of the selected cell-surface receptor whereby activity of the receptor can be monitored via transcription of the reporter genes.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art.

Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368); β-lactamase or GST.

Transcriptional control elements for use in the reporter gene constructs, or for modifying the genomic locus of an indicator gene include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is rapidly induced, generally within minutes, of contact between the cell surface protein and the effector protein that modulates the activity of the cell surface protein. Examples of such genes include, but are not limited to, the immediate early genes (see, Sheng et al. (1990) Neuron 4: 477-485), such as c-fos. Immediate early genes are genes that are rapidly induced upon binding of a ligand to a cell surface protein. The transcriptional control elements that are preferred for use in the gene constructs include transcriptional control elements from immediate early genes, elements derived from other genes that exhibit some or all of the characteristics of the immediate early genes, or synthetic elements that are constructed such that genes in operative linkage therewith exhibit such characteristics. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is

transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

Other promoters and transcriptional control elements, in addition to those described above, include the vasoactive intestinal peptide (VIP) gene promoter (cAMP responsive; Fink et al. (1988), Proc. Natl. Acad. Sci. 85:6662-6666); the somatostatin gene promoter (cAMP responsive; Montminy et al. (1986), Proc. Natl. Acad. Sci. 8.3:6682-6686); the proenkephalin promoter (responsive to cAMP, nicotinic agonists, and phorbol esters; Comb et al. (1986), Nature 323:353-356); the phosphoenolpyruvate carboxy-kinase gene promoter (cAMP responsive; Short et al. (1986), J. Biol. Chem. 261:9721-9726); the NGFI-A gene promoter (responsive to NGF, cAMP, and serum; Changelian et al. (1989). Proc. Natl. Acad. Sci. 86:377-381); and others that may be known to or prepared by those of skill in the art.
In the case of receptors which modulate cyclic AMP, a transcriptional based readout can be constructed using the cyclic AMP response element binding protein, CREB, which is a transcription factor whose activity is regulated by phosphorylation at a particular serine (S133). When this serine residue is phosphorylated, CREB binds to a recognition sequence known as a CRE (cAMP Responsive Element) found to the 5' of promotors known to be responsive to elevated cAMP levels. Upon binding of phosphorylated CREB to a CRE, transcription from this promoter is increased. Phosphorylation of CREB is seen in response to both increased cAMP levels and increased intracellular Ca levels. Increased cAMP levels result in activation of PKA, which in turn phosphorylates CREB and leads to binding to CRE and transcriptional activation. Increased intracellular calcium levels results in activation of calcium/calmodulin responsive kinase II (CaM kinase II). Phosphorylation of CREB by CaM kinase II is effectively the same as phosphorylation of CREB by PKA, and results in transcriptional activation of CRE containing promotors.

Therefore, a transcriptionally based readout can be constructed in cells containing a reporter gene whose expression is driven by a basal promoter

containing one or more CRE. Changes in the intracellular concentration of Ca^{++} (a result of alterations in the activity of the receptor upon engagement with a ligand) will result in changes in the level of expression of the reporter gene if: a) CREB is also co-expressed in the cell, and b) either an endogenous or heterologous CaM kinase phosphorylates CREB in response to increases in calcium or if an exogenously expressed CaM kinase II is present in the same cell. In other words, stimulation of PLC activity may result in phosphorylation of CREB and increased transcription from the CRE-construct, while inhibition of PLC activity may result in decreased transcription from the CRE-responsive construct.

As described in Bonni et al. (1993) *Science* 262:1575-1579, the observation that CNTF treatment of SK-N-MC cells leads to the enhanced interaction of STAT/p91 and STAT related proteins with specific DNA sequences suggested that these proteins might be key regulators of changes in gene expression that are triggered by CNTF. Consistent with this possibility is the finding that DNA sequence elements similar to the consensus DNA sequence required for STAT/p91 binding are present upstream of a number of genes previously found to be induced by CNTF (e.g., Human c-fos, Mouse c-fos, Mouse tis11, Rat junB, Rat SOD-1, and CNTF). Those authors demonstrated the ability of STAT/p91 binding sites to confer CNTF responsiveness to a non-responsive reporter gene. Accordingly, a reporter construct for use in the present invention for detecting signal transduction through STAT proteins, such as from cytokine receptors, can be generated by using -71 to +109 of the mouse c-fos gene fused to the bacterial chloramphenicol acetyltransferase gene (-71fosCAT) or other detectable marker gene. Induction by a cytokine receptor induces the tyrosine phosphorylation of STAT and STAT-related proteins, with subsequent translocation and binding of these proteins to the STAT-RE. This then leads to activation of transcription of genes containing this DNA element within their promoters.

In preferred embodiments, the reporter gene is a gene whose expression causes a phenotypic change that is screenable or selectable. If the change is selectable, the phenotypic change creates a difference in the growth or survival rate

between cells that express the reporter gene and those which do not. If the change is screenable, the phenotype change creates a difference in some detectable characteristic of the cells, by which the cells that express the marker may be distinguished from those which do not. Selection is preferable to screening in that it can provide a means for amplifying from the cell culture those cells that express a test antibody which is a receptor effector.

The marker gene is coupled to the receptor signaling pathway so that expression of the marker gene is dependent on activation of the receptor. This coupling may be achieved by operably linking the marker gene to a receptor-responsive promoter. The term "receptor-responsive promoter" indicates a promoter that is regulated by some product of the target receptor's signal transduction pathway.

Alternatively, the promoter may be one that is repressed by the receptor pathway, thereby preventing expression of a product that is deleterious to the cell. With a receptor repressed promoter, one screens for agonists by linking the promoter to a deleterious gene, and for antagonists, by linking it to a beneficial gene. Repression may be achieved by operably linking a receptor- induced promoter to a gene encoding mRNA which is antisense to at least a portion of the mRNA encoded by the marker gene (whether in the coding or flanking regions), so as to inhibit translation of that mRNA. Repression may also be obtained by linking a receptor-induced promoter to a gene encoding a DNA binding repressor protein, and incorporating a suitable operator site into the promoter or other suitable region of the marker gene.

The marker gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include β-galactosidase (Xgal, C_{12}FDG, Salmon-gal, Magenta-Gal (latter two from Biosynth Ag)), alkaline phosphatase, horseradish peroxidase, exo-glucanase (product of yeast exbl gene; nonessential, secreted); luciferase; bacterial green fluorescent protein; (human placental) secreted alkaline phosphatase (SEAP); and chloramphenicol transferase (CAT). Some of the above can be

engineered so that they are secreted (although not β-galactosidase). A preferred screenable marker gene is beta-galactosidase; yeast cells expressing the enzyme convert the colorless substrate Xgal into a blue pigment. Again, the promoter may be receptor-induced or receptor-inhibited.

In certain assays it may be desirable to use changes in growth in the screening procedure. For example, one of the consequences of activation of the pheromone signal pathway in wild-type yeast is growth arrest. If one is testing for an antagonist of a G protein-coupled receptor, such as a human receptor engineered into a yeast cell, this normal response of growth arrest can be used to select cells in which the pheromone response pathway is inhibited. That is, cells exposed to a test compound will be growth arrested if the compound is an agonist, but will grow normally if the compound is neutral or an antagonist. Thus, the growth arrest response can be used to advantage to discover compounds that function as agonists or antagonists. Moreover, the effect of growth arrest can provide a selective advantage in the presence of an agent that is cytotoxic to mitotic cells. For example, during the growth arrest window, the cytotoxic agent is added to the culture. Cells that proceed through the cell-cycle, e.g., that are not growth arrested, will be killed. At some time after the addition of the cytotoxic agent, it can be washed from the culture, and surviving cells permitted to proceed with proliferation. Cells that were arrested by the test compound will be enriched in the surviving population. However, in certain embodiments the growth arrest consequent to activation of the pheromone response pathway is an undesirable effect since cells that bind agonists stop growing while surrounding cells that fail to bind antibodies will continue to grow. The cells of interest, then, will be overgrown or their detection obscured by the background cells, confounding identification of agonistic antibodies. To overcome this problem the present invention teaches engineering the cell such that: 1) growth arrest does not occur as a result of exogenous signal pathway activation (e.g., by inactivating the FAR1 gene); and/or 2) a selective growth advantage is conferred by activating the pathway (e.g., by transforming an auxotrophic mutant

with a HIS3 gene under the control of a pheromone-responsive promoter, and applying selective conditions).

It is, of course, desirable that the exogenous receptor be exposed on a continuing basis to the antibodies. Unfortunately, this is likely to result in desensitization of the pheromone pathway to the stimulus. For example, the mating signal transduction pathway is known to become desensitized by several mechanisms including pheromone degradation and modification of the function of the receptor, G proteins, and/or downstream elements of the pheromone signal transduction by the products of the SST2, STE50, AFR1 (Konopka, J.B. (1993) *Mol. Cell. Biol.* 13:6876-6888) and SGV1, MSG5, and SIG1 genes. Selected mutations in these genes can lead to hypersensitivity to pheromone and an inability to adapt to the presence of pheromone. For example, introduction of mutations that interfere with function into strains expressing heterologous G protein-coupled receptors constitutes a significant improvement on wild type strains and enables the development of extremely sensitive bioassays for compounds that interact with the receptors. Other mutations e.g. STE50, sgv1, *bar1*, ste2, ste3, pik1, msg5, sig1, and aft1, have the similar effect of increasing the sensitivity of the bioassay. Thus desensitization may be avoided by mutating (which may include deleting) the SST2 gene so that it no longer produces a functional protein, or by mutating one of the other genes listed above.

If the endogenous homolog of the receptor is produced by the yeast cell, the assay will not be able to distinguish between antibodies which interact with the endogenous receptor and those which interact with the exogenous receptor. It is therefore desirable that the endogenous gene be deleted or otherwise rendered nonfunctional.

Suitable host cells for generating the target cells of subject assay include prokaryotes, yeast, or higher eukaryotic cells, including plant and animal cells, especially mammalian cells. Prokaryotes include gram-negative or gram-positive organisms. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman (1981) Cell 23:175) CV-1

cells (ATCC CCL 70), L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa, HEK-293, SWISS 3T3, and BHK cell lines.

If yeast cells are used, the yeast may be of any species which are cultivable and in which an exogenous receptor can be made to engage the appropriate signal transduction machinery of the host cell. Suitable species include *Kluyverei lactis, Schizosaccharomyces pombe*, and *Ustilaqo maydis; Saccharomyces cerevisiae* is preferred. Other yeasts which can be used in practicing the present invention are *Neurospora crassa, Aspergillus niger, Aspergillus nidulans, Pichia pastoris, Candida tropicalis, and Hansenula polymorpha.* The term "yeast", as used herein, includes not only yeast in a strictly taxonomic sense, i.e., unicellular organisms, but also yeast-like multicellular fungi or filamentous fungi.

The choice of appropriate host cell will also be influenced by the choice of detection signal. For instance, reporter constructs, as described below, can provide a selectable or screenable trait upon transcriptional activation (or inactivation) in response to a signal transduction pathway coupled to the target receptor. The reporter gene may be an unmodified gene already in the host cell pathway. It may be a host cell gene that has been operably linked to a "receptor-responsive" promoter. Alternatively, it may be a heterologous gene (e.g., a "reporter gene construct") that has been so linked. Suitable genes and promoters are discussed below. In other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium or phospholipid metabolism are quantitated. In yet other embodiments indicator genes can be used to detect receptor-mediated signaling.

Accordingly, it will be understood that to achieve selection or screening, the host cell must have an appropriate phenotype. For example, generating a pheromone-responsive chimeric HIS3 gene in a yeast that has a wild-type HIS3 gene would frustrate genetic selection. Thus, to achieve nutritional selection, an auxotrophic strain is wanted.

A variety of complementations for use in the subject assay can be constructed. Indeed, many yeast genetic complementation with mammalian signal

transduction proteins have been described in the art. For example, Mosteller et al. (1994) Mol Cell Biol 14:1104-12 demonstrates that human Ras proteins can complement loss of ras mutations in *S. cerevisiae*. Moreover, Toda et al. (1986) Princess Takamatsu Symp 17: 253-60 have shown that human ras proteins can complement the loss of RAS1 and RAS2 proteins in yeast, and hence are functionally homologous. Both human and yeast RAS proteins can stimulate the magnesium and guanine nucleotide-dependent adenylate cyclase activity present in yeast membranes. Ballester et al. (1989) Cell 59: 681-6 describe a vector to express the mammalian GAP protein in the yeast *S. cerevisiae*. When expressed in yeast, GAP inhibits the function of the human ras protein, and complements the loss of IRA1. IRA1 is a yeast gene that encodes a protein with homology to GAP and acts upstream of RAS. Mammalian GAP can therefore function in yeast and interact with yeast RAS. Wei et al. (1994) Gene 151: 279-84 describes that a human Ras-specific guanine nucleotide-exchange factor, Cdc25GEF, can complement the loss of CDC25 function in *S. cerevisiae*. Martegani et al. (1992) EMBO J 11: 2151-7 describe the cloning by functional complementation of a mouse cDNA encoding a homolog of CDC25, a *Saccharomyces cerevisiae* RAS activator. Vojtek et al. (1993) J Cell Sci 105: 777-85 and Matviw et al. (1992) Mol Cell Biol 12: 5033-40 describe how a mouse CAP protein, e.g., an adenylyl cyclase associated protein associated with ras-mediated signal transduction, can complement defects in *S. cerevisiae*. Papasavvas et al. (1992) Biochem Biophys Res Commun 184:1378-85 also suggest that inactivated yeast adenyl cyclase can be complemented by a mammalian adenyl cyclase gene. Hughes et al. (1993) Nature 364: 349-52 describe the complementation of byr1 in fission yeast by mammalian MAP kinase kinase (MEK). Parissenti et al. (1993) Mol Cell Endocrinol 98: 9-16 describes the reconstitution of bovine protein kinase C (PKC) in yeast. The Ca(2+)- and phospholipid-dependent Ser/Thr kinase PKC plays important roles in the transduction of cellular signals in mammalian cells. Marcus et al. (1995) PNAS 92: 6180-4 suggests the complementation of shk1 null mutations in *S. pombe* by the either the structurally related *S. cerevisiae* Ste20 or mammalian p65PAK protein kinases.

"Inactivation", with respect to genes of the host cell, means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved by deletion of the gene, mutation of the promoter so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive. Inactivation may be partial or total.

"Complementation", with respect to genes of the host cell, means that at least partial function of inactivated gene of the host cell is supplied by an exogenous nucleic acid. For instance, yeast cells can be "mammalianized", and even "humanized", by complementation of receptor and signal transduction proteins with mammalian homologs. To illustrate, inactivation of a yeast Byr2/Ste11 gene can be complemented by expression of a human MEKK gene.

C. Generations of Antibody Libraries

The variegated antibody libraries of the subject method can be generated by any of a number of methods, and, though not limited by, preferably exploit recent trends in the preparation of antibody libraries.

In one embodiment, after application of an immunization step, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known, and can be applied in the subject method, for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) Biotechniques 11: 152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al. (1991) Methods: Companion to Methods in Enzymology 2: 106-110). The ability to clone human immunoglobulin V-genes takes on special significance in light of advancements in creating human antibody repertoires in transgenic animals (see, for example, Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al.

(1991) *Eur J Immunol* 21:1323-1326; and Wood et al. PCT publication WO 91/00906).

In an illustrative embodiment, RNA is isolated from mature B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, such as those shown in Figures 1A and 1B (for mouse) or Figure 6 (for human), the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages.

Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

IV. Exemplary Uses

Because of the flexibility of the system, the subject method can be used in a broad range of applications, including for the selection of antibodies having effects on proliferation, differentiation, cell death, cell migration, etc. In preferred embodiments, the target used in the display mode is an extracellular component of a cell. However, it will be appreciated that the target for the subject method can be an intracellular component and, during the secretion mode, the system can be augmented with agents that promote the cellular uptake of the test antibodies.

In an illustrative embodiment, the subject method is utilized to identify antibodies that have antiproliferative activity with respect to one or more types of cells. For instance, in the display mode, the antibody library can be panned with the target cells for which an antiproliferative is desired in order to enrich for antibodies that

bind to that cell. At that stage, the antibody library can also be panned against one or more control cell lines in order to remove antibodies that bind the control cells. In this manner, the antibody library that is then tested in the secretion mode can be enriched for antibodies that selectively bind target cell (relative to the control cells). Thus, for example, the display mode can produce an antibody library enriched for antibodies that preferentially bind tumor cells relative to normal cells, preferentially bind p53- cells relative to p53+ cells, preferentially bind hair follicle cells relative to other epithelial cells, or exhibit any other differential binding characteristic.

In the secretion mode, the antibodies are tested for antiproliferative activity against the target cell using any of a number of techniques known in the art. For instance, BrdU or other nucleotide uptake can be measured as an indicator of proliferation. As above, the secretion mode can include negative controls in order to select for antibodies with specific antiproliferative activity.

In similar fashion, antibodies can be isolated from the library based on their ability to induce apoptosis or cell lysis, e.g., in a cell selective manner.

In yet another embodiment, the subject method can be used to identify antibodies with angiogenic or antiangiogenic activity. For instance, as illustrated in Figure 6, the antibody library can be enriched for antibodies that bind to endothelial cells but which do not bind to fibroblasts. The resulting sub-library can be screened for antibodies that inhibit capillary endothelial cell proliferation and/or endothelial cell migration. Antibodies scoring positive for one or both of these activities can also be tested for activity against other cell types, such as smooth muscle cells or fibroblasts, in order to select antibodies active only against endothelial cells.

In still another embodiment, the subject method can be used to identify anti-infective antibodies, e.g., which are active as anti-fungal or antibacterial agents.

In one embodiment, the assay of the present invention can be used for identifying effectors of a receptor protein or complex thereof. In general, the assay is characterized by the use of a test cell that includes a target receptor or ion channel protein whose signal transduction activity can be modulated by interaction with an

extracellular signal, the transduction activity being able to generate a detectable signal.

In general, such embodiments of the subject assay are characterized by the use of a mixture of cells expressing a target receptor protein or ion channel capable of transducing a detectable signal in the reagent cell. The receptor/channel protein can be either endogenous or heterologous. In combination with the disclosed detection means, a culture of the instant reagent cells will provide means for detecting agonists or antagonists of receptor function.

The ability of particular antibodies to modulate a signal transduction activity of the target receptor or channel can be scored for by detecting up- or down-regulation of the detection signal. For example, second messenger generation (e.g., GTPase activity, phospholipid hydrolysis, or protein phosphorylation patterns as examples) can be measured directly. Alternatively, the use of an indicator gene can provide a convenient readout. In other embodiments a detection means consists of an indicator gene. In any event, a statistically significant change in the detection signal can be used to facilitate identification of compounds that modulate receptor or ion channel activities.

By this method, antibodies that induce a signal pathway from a particular receptor or channel can be identified. If a test antibody does not appear to induce the activity of the receptor/channel protein, the assay may be repeated as described above, and modified by the introduction of a step in which the reagent cell is first contacted with a known activator of the target receptor/channel to induce signal transduction, and the test antibody can be assayed for its ability to inhibit the activated receptor/channel, e.g., to identify antagonists. In yet other embodiments, antibodies can be screened for ones that potentiate the response to a known activator of the receptor.

With respect to the receptor or ion channel, it may be endogenously expressed by the host cell, or it may be expressed from a heterologous gene that has been introduced into the cell. Methods for introducing heterologous DNA into eukaryotic cells are of course well known in the art and any such method may be

used. In addition, DNA encoding various receptor proteins is known to those of skill in the art or it may be cloned by any method known to those of skill in the art. In certain embodiments, such as when an exogenous receptor is expressed, it may be desirable to inactivate, such as by deletion, a homologous receptor present in the cell.

In particular, the assays can be used to test functional ligand-receptor or ligand-ion channel interactions for cell surface-localized receptors and channels. As described in more detail below, the subject assay can be used to identify effectors of, for example, G protein-coupled receptors, receptor tyrosine kinases, cytokine receptors, and ion channels. In certain embodiments the method described herein is used for identifying ligands for "orphan receptors" for which no ligand is known. In preferred embodiments, the receptor is a cell surface receptor, such as: a receptor tyrosine kinase, e.g., an EPH receptor; an ion channel; a cytokine receptor; a multisubunit immune recognition receptor; a chemokine receptor; a growth factor receptor; or a G-protein coupled receptor, such as a chemoattractant antibody receptor; a neuroantibody receptor; a light receptor; a neurotransmitter receptor; or a polypeptide hormone receptor.

Preferred G protein coupled receptors include α1A-adrenergic receptor, α 1B-adrenergic receptor, α2-adrenergic receptor, α2B-adrenergic receptor, β1-adrenergic receptor, β2-adrenergic receptor, β3-adrenergic receptor, m1 acetylcholine receptor (AChR), m2 AChR, m3 AChR, m4 AChR, m5 AChR, D1 dopamine receptor, D2 dopamine receptor, D3 dopamine receptor, D4 dopamine receptor, D5 dopamine receptor, A1 adenosine receptor, A2b adenosine receptor, 5-HT1a receptor, 5-HT1b receptor, 5HT1-like receptor, 5-HT1d receptor, 5HT1d-like receptor, 5HT1d beta receptor, substance K (neurokinin A) receptor, fMLP receptor, fMLP-like receptor, angiotensin II type 1 receptor, endothelin ETA receptor, endothelin ETB receptor, thrombin receptor, growth hormone-releasing hormone (GHRH) receptor, vasoactive intestinal antibody receptor, oxytocin receptor, somatostatin SSTR1 and SSTR2, SSTR3, cannabinoid receptor, follicle stimulating hormone (FSH) receptor, leutropin (LH/HCG) receptor, thyroid stimulating

hormone (TSH) receptor, thromboxane A2 receptor, platelet-activating factor (PAF) receptor, C5a anaphylatoxin receptor, Interleukin 8 (IL-8) IL-8RA, IL-8RB, Delta Opioid receptor, Kappa Opioid receptor, mip-1/RANTES receptor, Rhodopsin, Red opsin, Green opsin, Blue opsin, metabotropic glutamate mGluR1-6, histamine H2 receptor, ATP receptor, neuroantibody Y receptor, amyloid protein precursor receptor, insulin-like growth factor II receptor, bradykinin receptor, gonadotropin-releasing hormone receptor, cholecystokinin receptor, melanocyte-stimulating hormone receptor receptor, antidiuretic hormone receptor, glucagon receptor, and adrenocorticotropic hormone II receptor.

Preferred EPH receptors inlcude eph, elk, eck, sek, mek4, hek, hek2, eek, erk, tyro1, tyro4, tyro5, tyro6, tyro11, cek4, cek5, cek6, cek7, cek8, cek9, cek10, bsk, rtk1, rtk2, rtk3, myk1, myk2, ehk1, ehk2, pagliaccio, htk, erk, and nuk receptors.

A. Cytokine Receptors

In one embodiment the target receptor is a cytokine receptor. Cytokines are a family of soluble mediators of cell-to-cell communication that includes interleukins, interferons, and colony-stimulating factors. The characteristic features of cytokines lie in their functional redundancy and pleiotropy. Most of the cytokine receptors that constitute distinct superfamilies do not possess intrinsic protein tyrosine kinase domains, yet receptor stimulation usually invokes rapid tyrosine phosphorylation of intracellular proteins, including the receptors themselves. Many members of the cytokine receptor superfamily acitvate the Jak protein tyrosine kinase family, with resultant phosphorylation of the STAT transcriptional activator factors. IL-2, IL-7, IL-2 and Interferon γ have all been shown to activate Jak kinases (Frank et al (1995) *Proc Natl Acad Sci USA* 92:7779-7783); Scharfe et al. (1995) *Blood* 86:2077-2085); (Bacon et al. (1995) *Proc Natl Acad Sci USA* 92:7307-7311); and (Sakatsume et al (1995) *J. Biol Chem* 270:17528-17534). Events downstream of Jak phosphorylation have also been elucidated. For example, exposure of T lymphocytes to IL-2 has been shown to lead to the phosphorylation of signal transducers and activators of transcription (STAT) proteins STAT1α, STAT2β, and STAT3, as well as of two

STAT-related proteins, p94 and p95. The STAT proteins were found to translocate to the nucleus and to bind to a specific DNA sequence, thus suggesting a mechanism by which IL-2 may activate specific genes involved in immune cell function (Frank et al. *supra*). Jak3 is associated with the gamma chain of the IL-2, IL-4, and IL-7 cytokine receptors (Fujii et al. (1995) *Proc Natl Acad Sci* 92:5482-5486) and (Musso et al (1995) J Exp Med. 181:1425-1431). The Jak kinases have also been shown to be activated by numerous ligands that signal via cytokine receptors such as, growth hormone and erythropoietin and IL-6 (Kishimoto (1994) Stem cells Suppl 12:37-44).

Detection means that may be used for scoring in the present assay, in addition to direct detection of second messengers, such as by changes in phosphorylation, include reporter constructs or indicator genes that include transcriptional regulatory elements responsive to the STAT proteins. Described *infra*.

B. Multisubunit Immune Recognition Receptor (MIRR).

In another embodiment the receptor is a multisubunit receptor. Receptors can be comprised of multiple proteins referred to as subunits, one category of which is referred to as a multisubunit receptor is a multisubunit immune recognition receptor (MIRR). MIRRs include receptors having multiple noncovalently associated subunits and are capable of interacting with src-family tyrosine kinases. MIRRs can include, but are not limited to, B cell antigen receptors, T cell antigen receptors, Fc receptors and CD22. One example of an MIRR is an antigen receptor on the surface of a B cell. To further illustrate, the MIRR on the surface of a B cell comprises membrane-bound immunoglobulin (mIg) associated with the subunits Ig-α and Ig-β or Ig-γ, which forms a complex capable of regulating B cell function when bound by antigen. An antigen receptor can be functionally linked to an amplifier molecule in a manner such that the amplifier molecule is capable of regulating gene transcription. Src-family tyrosine kinases are enzymes capable of phosphorylating tyrosine residues of a target molecule. Typically, a src-family tyrosine kinase contains one or more binding domains and a kinase domain. A binding domain of a src-family

tyrosine kinase is capable of binding to a target molecule and a kinase domain is capable of phosphorylating a target molecule bound to the kinase. Members of the src family of tyrosine kinases are characterized by an N-terminal unique region followed by three regions that contain different degrees of homology among all the members of the family. These three regions are referred to as src homology region 1 (SH1), src homology region 2 (SH2) and src homology region 3 (SH3). Both the SH2 and SH3 domains are believed to have protein association functions important for the formation of signal transduction complexes. The amino acid sequence of an N-terminal unique region, varies between each src-family tyrosine kinase. An N-terminal unique region can be at least about the first 40 amino acid residues of the N-terminus of a src-family tyrosine kinase.

Syk-family kinases are enzymes capable of phosphorylating tyrosine residues of a target molecule. Typically, a syk-family kinase contains one or more binding domains and a kinase domain. A binding domain of a syk-family tyrosine kinase is capable of binding to a target molecule and a kinase domain is capable of phosphorylating a target molecule bound to the kinase. Members of the syk-family of tyrosine kinases are characterized by two SH2 domains for protein association function and a tyrosine kinase domain.

A primary target molecule is capable of further extending a signal transduction pathway by modifying a second messenger molecule. Primary target molecules can include, but are not limited to, phosphatidylinositol 3-kinase (PI-3K), $P21^{ras}$GAPase-activating protein and associated P190 and P62 protein, phospholipases such as PLCγ1 and PLCγ2, MAP kinase, Shc and VAV. A primary target molecule is capable of producing second messenger molecule that is capable of further amplifying a transduced signal. Second messenger molecules include, but are not limited to diacylglycerol and inositol 1,4,5-triphosphate (IP3). Second messenger molecules are capable of initiating physiological events that can lead to alterations in gene transcription. For example, production of IP3 can result in release of intracellular calcium, which can then lead to activation of calmodulin kinase II, which can then lead to serine phosphorylation of a DNA binding protein referred to

as ets-1 proto-onco-protein. Diacylglycerol is capable of activating the signal transduction protein, protein kinase C, which affects the activity of the AP1 DNA binding protein complex. Signal transduction pathways can lead to transcriptional activation of genes such as c-fos, egr-1, and c-myc.

Shc can be thought of as an adaptor molecule. An adaptor molecule comprises a protein that enables two other proteins to form a complex (e.g., a three-molecule complex). Shc protein enables a complex to form that includes Grb2 and SOS. Shc comprises an SH2 domain that is capable of associating with the SH2 domain of Grb2.

Molecules of a signal transduction pathway can associate with one another using recognition sequences. Recognition sequences enable specific binding between two molecules. Recognition sequences can vary depending upon the structure of the molecules that are associating with one another. A molecule can have one or more recognition sequences, and as such can associate with one or more different molecules.

Signal transduction pathways for MIRR complexes are capable of regulating the biological functions of a cell. Such functions can include, but are not limited to the ability of a cell to grow, to differentiate and to secrete cellular products. MIRR-induced signal transduction pathways can regulate the biological functions of specific types of cells involved in particular responses by an animal, such as immune responses, inflammatory responses and allergic responses. Cells involved in an immune response can include, for example, B cells, T cells, macrophages, dendritic cells, natural killer cells, and plasma cells. Cells involved in inflammatory responses can include, for example, basophils, mast cells, eosinophils, neutrophils and macrophages. Cells involved in allergic responses can include, for example mast cells, basophils, B cells, T cells, and macrophages.

In exemplary embodiments of the subject assay, the detection signal is a second messengers, such as a phosphorylated src-like protein, includes reporter constructs or indicator genes which include transcriptional regulatory elements such as serum response element (SRE), 12-O-tetradecanoyl-phorbol-13-acetate response

element, cyclic AMP response element, c-fos promoter, or a CREB-responsive element.

C. Receptor tyrosine kinases.

In still another embodiment, the target receptor is a receptor tyrosine kinase. The receptor tyrosine kinases can be divided into five subgroups on the basis of structural similarities in their extracellular domains and the organization of the tyrosine kinase catalytic region in their cytoplasmic domains. Sub-groups I (epidermal growth factor (EGF) receptor-like), II (insulin receptor-like) and the eph/eck family contain cysteine-rich sequences (Hirai et al., (1987) *Science* 238:1717-1720 and Lindberg and Hunter, (1990) *Mol. Cell. Biol.* 10:6316-6324). The functional domains of the kinase region of these three classes of receptor tyrosine kinases are encoded as a contiguous sequence (Hanks et al. (1988) *Science* 241:42-52). Subgroups III (platelet-derived growth factor (PDGF) receptor-like) and IV (the fibroblast growth factor (FGF) receptors) are characterized as having immunoglobulin (Ig)-like folds in their extracellular domains, as well as having their kinase domains divided in two parts by a variable stretch of unrelated amino acids (Yanden and Ullrich (1988) *supra* and Hanks et al. (1988) *supra*).

The family with by far the largest number of known members is the EPH family. Since the description of the prototype, the EPH receptor (Hirai et al. (1987) *Science* 238:1717-1720), sequences have been reported for at least ten members of this family, not counting apparently orthologous receptors found in more than one species. Additional partial sequences, and the rate at which new members are still being reported, suggest the family is even larger (Maisonpierre et al. (1993) *Oncogene* 8:3277-3288; Andres et al. (1994) *Oncogene* 9:1461-1467; Henkemeyer et al. (1994) *Oncogene* 9:1001-1014; Ruiz et al. (1994) *Mech Dev* 46:87-100; Xu et al. (1994) *Development* 120:287-299; Zhou et al. (1994) *J Neurosci Res* 37:129-143; and references in Tuzi and Gullick (1994) *Br J Cancer* 69:417-421). Remarkably, despite the large number of members in the EPH family, all of these molecules were identified as orphan receptors without known ligands.

The expression patterns determined for some of the EPH family receptors have implied important roles for these molecules in early vertebrate development. In particular, the timing and pattern of expression of *sek*, *mek4* and some of the other receptors during the phase of gastrulation and early organogenesis has suggested functions for these receptors in the important cellular interactions involved in patterning the embryo at this stage (Gilardi-Hebenstreit et al. (1992) *Oncogene* 7:2499-2506; Nieto et al. (1992) *Development* 116:1137-1150; Henkemeyer et al., *supra*; Ruiz et al., *supra*; and Xu et al., *supra*). *Sek*, for example, shows a notable early expression in the two areas of the mouse embryo that show obvious segmentation, namely the somites in the mesoderm and the rhombomeres of the hindbrain; hence the name *sek*, for segmentally expressed kinase (Gilardi-Hebenstreit et al., *supra*; Nieto et al., *supra*). As in Drosophila, these segmental structures of the mammalian embryo are implicated as important elements in establishing the body plan. The observation that Sek expression precedes the appearance of morphological segmentation suggests a role for *sek* in forming these segmental structures, or in determining segment-specific cell properties such as lineage compartmentation (Nieto et al., *supra*). Moreover, EPH receptors have been implicated, by their pattern of expression, in the development and maintenance of nearly every tissue in the embryonic and adult body. For instance, EPH receptors have been detected throughout the nervous system, the testes, the cartilaginous model of the skeleton, tooth primordia, the infundibular component of the pituitary, various epithelia tissues, lung, pancreas, liver and kidney tissues. Observations such as this have been indicative of important and unique roles for EPH family kinases in development and physiology, but further progress in understanding their action has been severely limited by the lack of information on their ligands.

As used herein, the terms "EPH receptor" or "EPH-type receptor" refer to a class of receptor tyrosine kinases, comprising at least eleven paralogous genes, though many more orthologs exist within this class, e.g., homologs from different species. EPH receptors, in general, are a discrete group of receptors related by homology and easily reconizable, e.g., they are typically characterized by an

extracellular domain containing a characteristic spacing of cysteine residues near the N-terminus and two fibronectin type III repeats (Hirai et al. (1987) *Science* 238:1717-1720; Lindberg et al. (1990) *Mol. Cell Biol.* 10:6316-6324; Chan et al. (1991) *Oncogene* 6:1057-1061; Maisonpierre et al. (1993) *Oncogene* 8:3277-3288; Andres et al. (1994) *Oncogene* 9:1461-1467; Henkemeyer et al. (1994) *Oncogene* 9:1001-1014; Ruiz et al. (1994) *Mech Dev* 46:87-100; Xu et al. (1994) *Development* 120:287-299; Zhou et al. (1994) *J Neurosci Res* 37:129-143; and references in Tuzi and Gullick (1994) *Br J Cancer* 69:417-421). Exemplary EPH receptors include the *eph, elk, eck, sek, mek4, hek, hek2, eek, erk, tyro1, tyro4, tyro5, tyro6, tyro11, cek4, cek5, cek6, cek7, cek8, cek9, cek10, bsk, rtk1, rtk2, rtk3, myk1, myk2, ehk1, ehk2, pagliaccio, htk, erk,* and *nuk* receptors. The term "EPH receptor" refers to the membrane form of the receptor protein, as well as soluble extracellular fragments that retain the ability to bind the ligand of the present invention.

In exemplary embodiments, the detection signal is provided by detecting phosphorylation of intracellular proteins, e.g., MEKKs, MEKs, or Map kinases, or by the use of reporter constructs or indicator genes that include transcriptional regulatory elements responsive to c-fos and/or c-jun, as described *infra.*

D. G Protein-Coupled Receptors.

One family of signal transduction cascades found in eukaryotic cells utilizes heterotrimeric "G proteins." Many different G proteins are known to interact with receptors. G protein signaling systems include three components: the receptor itself, a GTP-binding protein (G protein), and an intracellular target protein.
The cell membrane acts as a switchboard. Messages arriving through different receptors can produce a single effect if the receptors act on the same type of G protein. On the other hand, signals activating a single receptor can produce more than one effect if the receptor acts on different kinds of G proteins, or if the G proteins can act on different effectors.

In their resting state, the G proteins, which consist of alpha (α), beta (β) and gamma (γ) subunits, are complexed with the nucleotide guanosine diphosphate (GDP) and are in contact with receptors. When a hormone or other first messenger

binds to receptor, the receptor changes conformation and this alters its interaction with the G protein. This spurs the α subunit to release GDP, and the more abundant nucleotide guanosine triphosphate (GTP), replaces it, activating the G protein. The G protein then dissociates to separate the α subunit from the still complexed beta and gamma subunits. Either the G α subunit, or the Gβγ complex, depending on the pathway, interacts with an effector. The effector (which is often an enzyme) in turn converts an inactive precursor molecule into an active "second messenger," which may diffuse through the cytoplasm, triggering a metabolic cascade. After a few seconds, the Gα converts the GTP to GDP, thereby inactivating itself. The inactivated Gα may then reassociate with the Gβγ complex.

Hundreds, if not thousands, of receptors convey messages through heterotrimeric G proteins, of which at least 17 distinct forms have been isolated. Although the greatest variability has been seen in the α subunit, several different β and γ structures have been reported. There are, additionally, several different G protein-dependent effectors.

Most G protein-coupled receptors are comprised of a single protein chain that is threaded through the plasma membrane seven times. Such receptors are often referred to as seven-transmembrane receptors (STRs). More than a hundred different STRs have been found, including many distinct receptors that bind the same ligand, and there are likely many more STRs awaiting discovery.

In addition, STRs have been identified for which the natural ligands are unknown; these receptors are termed "orphan" G protein-coupled receptors, as described above. Examples include receptors cloned by Neote et al. (1993) *Cell* 72, 415; Kouba et al. *FEBS Lett.* (1993) 321, 173; Birkenbach et al.(1993) *J. Virol.* 67, 2209.

The "exogenous receptors" of the present invention may be any G protein-coupled receptor that is exogenous to the cell that is to be genetically engineered for the purpose of the present invention. This receptor may be a plant or animal cell receptor. Screening for binding to plant cell receptors may be useful in the development of, e.g., herbicides. In the case of an animal receptor, it may be of

invertebrate or vertebrate origin. If an invertebrate receptor, such as an insect receptor, is employed, the assay can be used to facilitate development of insecticides. The receptor may also be a vertebrate, more preferably a mammalian, still more preferably a human, receptor. The exogenous receptor is also preferably a seven transmembrane segment receptor.

Known ligands for G protein coupled receptors include: purines and nucleotides, such as adenosine, cAMP, ATP, UTP, ADP, melatonin and the like; biogenic amines (and related natural ligands), such as 5-hydroxytryptamine, acetylcholine, dopamine, adrenaline, histamine, noradrenaline, tyramine/octopamine and other related compounds; antibodies such as adrenocorticotrophic hormone (acth), melanocyte stimulating hormone (msh), melanocortins, neurotensin (nt), bombesin and related antibodies, endothelins, cholecystokinin, gastrin, neurokinin b (nk3), invertebrate tachykinin-like antibodies, substance k (nk2), substance p (nk1), neuroantibody y (npy), thyrotropin releasing-factor (trf), bradykinin, angiotensin ii, beta-endorphin, c5a anaphylatoxin, calcitonin, chemokines (also called intercrines), corticotrophic releasing factor (crf), dynorphin, endorphin, fmlp and other formylated antibodies, follitropin (fsh), fungal mating pheremones, galanin, gastric inhibitory polypeptide receptor (gip), glucagon-like antibodies (glps), glucagon, gonadotropin-releasing hormone (gnrh), growth hormone releasing hormone(ghrh), insect diuretic hormone, interleukin-8, leutropin (lh/hcg), met-enkephalin, opioid antibodies, oxytocin, parathyroid hormone (pth) and pthrp, pituitary adenylyl cyclase activiating antibody (pacap), secretin, somatostatin, thrombin, thyrotropin (tsh), vasoactive intestinal antibody (vip), vasopressin, vasotocin; eicosanoids such as ip-prostacyclin, pg-prostaglandins, tx-thromboxanes; retinal-based compounds such as vertebrate 11-cis retinal, invertebrate 11-cis retinal and other related compounds; lipids and lipid-based compounds such as cannabinoids, anandamide, lysophosphatidic acid, platelet activating factor, leukotrienes and the like; excitatory amino acids, and ions such as calcium ions and glutamate.

Suitable examples of G-protein coupled receptors include, but are not limited to, dopaminergic, muscarinic cholinergic, α-adrenergic, β-adrenergic, opioid

(including delta and mu), cannabinoid, serotoninergic, and GABAergic receptors. Preferred receptors include the 5HT family of receptors, dopamine receptors, C5a receptor and FPRL-1 receptor, cyclo-histidyl-proline-diketoplperazine receptors, melanocyte-stimulating hormone release-inhibiting factor receptor, and receptors for neurotensin, thyrotropin-releasing hormone, calcitonin, cholecytokinin-A, neurokinin-2, histamine-3, cannabinoid, melanocortin, or adrenomodulin, neuroantibody-Y1 or galanin. Other suitable receptors are listed in the art. The term "receptor," as used herein, encompasses both naturally occurring and mutant receptors.

Many of these G protein-coupled receptors, like the yeast α- and β-factor receptors, contain seven hydrophobic amino acid-rich regions, which are assumed to lie within the plasma membrane. Specific human G protein-coupled STRs for which genes have been isolated and for which expression vectors could be constructed include those listed herein and others known in the art. Thus, the gene would be operably linked to a promoter functional in the cell to be engineered and to a signal sequence that also functions in the cell. For example in the case of yeast, suitable promoters include Ste2, Ste3 and gal10. Suitable signal sequences include those of Ste2, Ste3 and of other genes that encode proteins secreted by yeast cells. Preferably, when a yeast cell is used, the codons of the gene would be optimized for expression in yeast. See Hoekema et al., (1987) *Mol. Cell. Biol.*, 7:2914-24; Sharp, et al., (1986)14:5125-43.

The homology of STRs is discussed in Dohlman et al., *Ann. Rev. Biochem.*, (1991) 60:653-88. When STRs are compared, a distinct spatial pattern of homology is discernible. The transmembrane domains are often the most similar, whereas the N- and C-terminal regions, and the cytoplasmic loop connecting transmembrane segments V and VI are more divergent.

The functional significance of different STR regions has been studied by introducing point mutations (both substitutions and deletions) and by constructing chimeras of different but related STRs. Synthetic antibodies corresponding to

individual segments have also been tested for activity. Affinity labeling has been used to identify ligand-binding sites.

It is conceivable that when the host cell is a yeast cell, a foreign receptor will fail to functionally integrate into the yeast membrane, and there interact with the endogenous yeast G protein. More likely, either the receptor will need to be modified (e.g., by replacing its V-VI loop with that of the yeast STE2 or STE3 receptor), or a compatible G protein should be provided.

If the wild-type exogenous G protein-coupled receptor cannot be made functional in yeast, it may be mutated for this purpose. A comparison would be made of the amino acid sequences of the exogenous receptor and of the yeast receptors, and regions of high and low homology identified. Trial mutations would then be made to distinguish regions involved in ligand or G protein binding, from those necessary for functional integration in the membrane. The exogenous receptor would then be mutated in the latter region to more closely resemble the yeast receptor, until functional integration was achieved. If this were insufficient to achieve functionality, mutations would next be made in the regions involved in G protein binding. Mutations would be made in regions involved in ligand binding only as a last resort, and then an effort would be made to preserve ligand binding by making conservative substitutions whenever possible.

Preferably, the yeast genome is modified so that it is unable to produce the yeast receptors that are homologous to the exogenous receptors in functional form. Otherwise, a positive assay score might reflect the ability of an antibody to activate the endogenous G protein-coupled receptor, and not the receptor of interest.

(i). Chemoattractant receptors

The N-formyl antibody receptor is a classic example of a calcium-mobilizing G protein-coupled receptor expressed by neutrophils and other phagocytic cells of the mammalian immune system (Snyderman et al. (1988) *In Inflammation: Basic Principles and Clinical Correlates*, pp. 309-323). N-formyl antibodies of bacterial origin bind to the receptor and engage a complex activation program that results in directed cell movement, release of inflammatory granule contents, and activation of

a latent NADPH oxidase that is important for the production of metabolites of molecular oxygen. This pathway initiated by receptor-ligand interaction is critical in host protection from pyrogenic infections. Similar signal transduction occurs in response to the inflammatory antibodies C5a and IL-8.

Two other formyl antibody receptor-like (FPRL) genes have been cloned based on their ability to hybridize to a fragment of the NFPR cDNA coding sequence. These have been named FPRL1 (Murphy et al. (1992) *J. Biol Chem.* 267:7637-7643) and FPRL2 (Ye et al. (1992) *Biochem Biophys Res. Comm.* 184:582-589). FPRL2 was found to mediate calcium mobilization in mouse fibroblasts transfected with the gene and exposed to formyl antibody. In contrast, although FPRL1 was found to be 69% identical in amino acid sequence to NFPR, it did not bind prototype N-formyl antibodies ligands when expressed in heterologous cell types. This lead to the hypothesis of the existence of an as yet unidentified ligand for the FPRL1 orphan receptor (Murphy et al. *supra*).

(ii.) G proteins

In the case of an exogenous G-protein coupled receptor, the yeast cell must be able to produce a G protein which is activated by the exogenous receptor, and which can in turn activate the yeast effector(s). The art suggests that the endogenous yeast Gα subunit (e.g., GPA) will be often be sufficiently homologous to the "cognate" Gα subunit which is natively associated with the exogenous receptor for coupling to occur. More likely, it will be necessary to genetically engineer the yeast cell to produce a foreign Gα subunit that can properly interact with the exogenous receptor. For example, the Gα subunit of the yeast G protein may be replaced by the Gα subunit natively associated with the exogenous receptor.

Dietzel and Kurjan, (1987) *Cell,* 50:1001) demonstrated that rat Gαs functionally coupled to the yeast Gβγ complex. However, rat Gαi2 complemented only when substantially overexpressed, while Gα0 did not complement at all. Kang, et al., *Mol. Cell. Biol.*, (1990)10:2582). Consequently, with some foreign Gα subunits, it is not feasible to simply replace the yeast Gα.

If the exogenous G protein coupled receptor is not adequately coupled to yeast Gβγ by the Gα subunit natively associated with the receptor, the Gα subunit may be modified to improve coupling. These modifications often will take the form of mutations that increase the resemblance of the Gα subunit to the yeast Gα while decreasing its resemblance to the receptor-associated Gα. For example, a residue may be changed so as to become identical to the corresponding yeast Gα residue, or to at least belong to the same exchange group of that residue. After modification, the modified Gα subunit might or might not be "substantially homologous" to the foreign and/or the yeast Gα subunit.

The modifications are preferably concentrated in regions of the Gα that are likely to be involved in Gβγ binding. In some embodiments, the modifications will take the form of replacing one or more segments of the receptor-associated Gα with the corresponding yeast Gα segment(s), thereby forming a chimeric Gα subunit. (For the purpose of the appended claims, the term "segment" refers to three or more consecutive amino acids.) In other embodiments, point mutations may be sufficient. This chimeric Gα subunit will interact with the exogenous receptor and the yeast Gβγ complex, thereby permitting signal transduction. While use of the endogenous yeast Gβγ is preferred, if a foreign or chimeric Gβγ is capable of transducing the signal to the yeast effector, it may be used instead.

V. Pharmaceutical Preparations of Identified Agents

After identifying certain test antibodies in the subject assay ,e.g. as potential surrogate ligands, or receptor antagonists, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected antibodies both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, antibodies identified in the subject assay, or peptidomimetics thereof, can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

The antibodies selected in the subject assay, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol,

propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the antibody can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

V. Pharmaceutical Preparations of Identified Agents

After identifying certain test antibodies in the subject assay, e.g., as potential surrogate ligands, or receptor antagonists, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected antibodies both *in vitro*

and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, antibodies identified in the subject assay, or peptidomimetics thereof, can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

The antibodies selected in the subject assay, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the antibody can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not

exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific method and reagents described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Claims:

1. A method for generating an antibody with a selected biological activity, comprising:
   (i) providing an antibody display library comprising a variegated population of test antibodies expressed on the surface of a population of display packages;
   (ii) in a display mode, isolating, from the antibody display library, a sub-population of display packages enriched for test antibodies which have a desired binding specificity and/or affinity for a cell or a component thereof;
   (iii) in a secretion mode, simultaneously expressing the enriched test antibody sub-population under conditions wherein the test antibodies are secreted and are free of the display packages; and
   (iv) assessing the ability of the secreted test antibodies to regulate a biological process in a target cell.
2. The method of claim 1, wherein the antibody display library is a phage display library.
3. The method of claim 2, wherein the display packages of the phage display library are phage particles selected from M13, f1, fd, If1, Ike, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, ϕX-174, MS2 and f2.
4. The method of claim 2, wherein the phage display library is generated with a filamentous bacteriophage specific for Escherichia coli and the phage coat protein is coat protein III or coat protein VIII.
5. The method of claim 4, wherein the filamentous bacteriophage is selected from M13, fd, and f1.
6. The method of claim 1, wherein the antibody display library is a bacterial cell-surface display library or a spore display library.
7. The method of claim 2, wherein test antibodies are enriched from the antibody display library in the display mode by a differential binding means

comprising affinity separation of test antibodies which specifically bind the cell or component thereof from test antibodies which do not.

8. The method of claim 7, wherein the differential binding means comprises panning the antibody display library on whole cells.

9. The method of claim 7, wherein the differential binding means comprises an affinity chromatographic means in which a component of a cell is provided as part of an insoluble matrix.

10. The method of claim 9, wherein the insoluble matrix comprises a cell surface protein attached to a polymeric support.

11. The method of claim 7, wherein the differential binding means comprises immunoprecipitating the display packages.

12. The method of claim 1, wherein the display mode enriches for test antibodies that bind to a cell-type specific marker.

13. The method of claim 1, wherein the display mode enriches for test antibodies that bind to a cell surface receptor protein.

14. The method of claim 13, wherein the receptor protein is a G-protein coupled receptor.

15. The method of claim 14, wherein the G-protein coupled receptor is selected from: a chemoattractant antibody receptor, a neuroantibody receptor, a light receptor, a neurotransmitter receptor, a cyclic AMP receptor, and a polypeptide hormone receptor.

16. The method of claim 14, wherein the G-protein coupled receptor is selected from: α1A-adrenergic receptor, α1B-adrenergic receptor, α2-adrenergic receptor, α2B-adrenergic receptor, β1-adrenergic receptor, β2- adrenergic receptor, β3-adrenergic receptor, m1 acetylcholine receptor (AChR), m2 AChR, m3 AChR, m4 AChR, m5 AChR, D1 dopamine receptor, D2 dopamine receptor, D3 dopamine receptor, D4 dopamine receptor, D5 dopamine receptor, A1 adenosine receptor, A2b adenosine receptor, 5-HT1a, 5-HT1b, 5HT1-like, 5-HT1d, 5HT1d-like, 5HT1d beta, substance K (neurokinin A), fMLP receptor, fMLP-like receptor, angiotensin II type 1,

endothelin ETA, endothelin ETB, thrombin, growth hormone-releasing hormone (GHRH), vasoactive intestinal antibody, oxytocin, somatostatin SSTR1 and SSTR2, SSTR3, cannabinoid, follicle stimulating hormone (FSH), leutropin (LH/HCG), thyroid stimulating hormone (TSH), thromboxane A2, platelet-activating factor (PAF), C5a anaphylatoxin, Interleukin 8 (IL-8) IL-8RA, IL-8RB, Delta Opioid, Kappa Opioid, mip-1/RANTES, Rhodopsin, Red opsin, Green opsin, Blue opsin, metabotropic glutamate mGluR1-6, histamine H2, ATP, neuroantibody Y, amyloid protein precursor, insulin-like growth factor II, bradykinin, gonadotropin-releasing hormone, cholecystokinin, melanocyte stimulating hormone receptor, antidiuretic hormone receptor, glucagon receptor, and adrenocorticotropic hormone II.

17. The method of claim 13, wherein the receptor protein is a receptor tyrosine kinase.

18. The method of claim 17, wherein the receptor tyrosine kinase is an EPH receptor.

19. The method of claim 18, wherein the receptor is selected from: eph, elk, eck, sek, mek4, hek, hek2, eek, erk, tyro1, tyro4, tyro5, tyro6, tyro11, cek4, cek5, cek6, cek7, cek8, cek9, cek10, bsk, rtk1, rtk2, rtk3, myk1, myk2, ehk1, ehk2, pagliaccio, htk, erk and nuk receptors.

20. The method of claim 13, wherein the receptor protein is a cytokine receptor.

21. The method of claim 13, wherein the receptor protein is an MIRR receptor.

22. The method of claim 1, wherein the antibody display library includes at least 103 different test antibodies.

23. The method of claim 1, wherein the test antibodies are single chain antibodies (scFv).

24. The method of claim 1, wherein each of the test antibodies are encoded by a chimeric gene comprising (i) a coding sequence for the test antibody, (ii) a coding sequence for a surface protein of the display package for displaying the test antibodies on the surface of a population of display packages, and

(iii) RNA splice sites flanking the coding sequence for the surface protein, wherein, in the display mode, the chimeric gene is expressed as fusion protein including the test antibody and the surface protein, whereas in the secretion mode, the test antibody is expressed without the surface protein as a result of the coding sequence for the surface protein being removed by RNA splicing.

25. The method of claim 1, wherein the test antibodies are expressed by a eukaryotic cell in the secretion mode.

26. The method of claim 25, wherein the eukaryotic cell is a mammalian cell.

27. The method of claim 1, wherein the target cell is a eukaryotic cell.

28. The method of claim 27, wherein the eukaryotic cell is a mammalian cell.

29. The method of claim 28, wherein the mammalian cell is a human cell.

30. The method of claim 1, wherein the biological process includes a change in cell proliferation, cell differentiation or cell death.

31. The method of claim 1, wherein the biological process is detected by changes in intracellular calcium mobilization.

32. The method of claim 1, wherein the biological process is detected by changes in intracellular protein phosphorylation.

33. The method of claim 1, wherein the biological process is detected by changes in phospholipid metabolism.

34. The method of claim 1, wherein the biological process is detected by changes in expression of cell-specific marker genes.

35. The method of claim 13, wherein the target cell further comprises a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the signal transduction activity of the cell surface receptor protein, expression of the reporter gene providing the detectable signal.

36. The method of claim 35, wherein the reporter gene encodes a gene product that gives rise to a detectable signal selected from color, fluorescence,

luminescence, cell viability relief of a cell nutritional requirement, cell growth, and drug resistance.

37. The method of claim 36, wherein the reporter gene encodes a gene product selected from chloramphenicol acetyl transferase, beta-galactosidase and secreted alkaline phosphatase.

38. The method of claim 36, wherein the reporter gene encodes a gene product that confers a growth signal.

39. The method of claim 1, wherein the secretion mode includes expression of the test antibodies by a host cell co-cultured with the target cell.

40. The method of claim 39, wherein the co-cultured host and target cells are separated by a membrane which is permeable to the test antibody.

41. The method of claim 1, wherein the secretion mode comprises assessing the ability of the secreted test antibodies to inhibit the biological activity of an exogenously added compound on the target cells.

42. The method of claim 1, wherein in step (ii), display packages which bind to endothelial cells are isolated, and in step (iv), the ability of the secreted test antibodies to inhibit proliferation of endothelial cells is assessed.

43. The method of claim 43, wherein in step (iv), the ability of the secreted test antibodies to inhibit proliferation of endothelial cells in the presence of an angiogenic amount of an endogenous growth factor is assessed.

44. The method of claim 1, further comprising formulating, with a pharmaceutically acceptable carrier, one or more test antibodies that regulate the biological process in the target cell.

45. The method of claim 1, further comprising converting into peptidomimetics one or more test antibodies that regulate the biological process in the target cell.

46. The method of claim 45, further comprising formulating, with a pharmaceutically acceptable carrier, one or more peptidomimetics of one or more test antibodies that regulate the biological process in the target cell.

47. An antibody display library enriched for test antibodies having a desired binding specificity and/or affinity for a cell or a component thereof and which regulate a biological process in a target cell.

48. A vector comprising a chimeric gene for a chimeric protein, which chimeric gene comprises (i) a coding sequence for a test antibody, (ii) a coding sequence for a surface protein of a display package, and (iii) RNA splice sites flanking the coding sequence for the surface protein, wherein,

in a display mode, the chimeric gene is expressed as a fusion protein including the test antibody and the surface protein such that the test antibody can be displayed on the surface of a population of display packages,

whereas in the secretion mode, the test antibody is expressed without the surface protein as a result of the coding sequence for the surface protein being removed by RNA splicing.

49. The vector of claim 48, wherein the chimeric gene further comprises a secretion signal sequence for secretion of the test antibody in the secretion mode.

50. The vector of claim 49, wherein the secretion signal sequence causes secretion of the test antibody from eukaryotic cells.

51. The vector of claim 50, wherein the eukaryotic cells are mammalian cells.

52. The vector of claim 48, wherein the display package is a phage.

53. The vector of claim 52, wherein the phage is selected from M13, f1, fd, If1, Ike, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, ϕX-174, MS2 and f2.

54. The vector of claim 52, wherein the phage is a filamentous bacteriophage specific for Escherichia coli and the surface protein is coat protein III or coat protein VIII.

55. The vector of claim 54, wherein the filamentous bacteriophage is selected from M13, fd, and f1.

56. A vector library, each vector comprising a chimeric gene for a chimeric protein, which chimeric gene comprises (i) a coding sequence for a test

antibody, (ii) a coding sequence for a surface protein of a display package, and (iii) RNA splice sites flanking the coding sequence for the surface protein, wherein,

in a display mode, the chimeric gene is expressed as fusion protein including the test antibody and the surface protein such that the test antibody can be displayed on the surface of a population of display packages,

whereas in the secretion mode, the test antibody is expressed without the surface protein as a result of the coding sequence for the surface protein being removed by RNA splicing,

the vector library collectively encodes a variegated population of test antibodies.

57. The vector library of claim 56, wherein the chimeric gene further comprises a secretion signal sequence for secretion of the test antibody in the secretion mode.

58. The vector library of claim 57, wherein the secretion signal sequence causes secretion of the test antibody from eukaryotic cells.

59. The vector library of claim 58, wherein the eukaryotic cells are mammalian cells.

60. The vector library of claim 56, wherein the display package is a phage.

61. The vector library of claim 60, wherein the phage is selected from M13, f1, fd, If1, Ike, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, ϕX-174, MS2 and f2.

62. The vector library of claim 56, wherein the phage is a filamentous bacteriophage specific for Escherichia coli and the surface protein is coat protein III or coat protein VIII.

63. The vector library of claim 62, wherein the filamentous bacteriophage is selected from M13, fd, and f1.

64. The vector library of claim 56, wherein the vector library collectively encodes at least 103 different test antibodies.

65. The vector library of claim 56, wherein the test antibodies are single chain antibodies (scFv).

66. A cell composition comprising a population of cells containing the vector library of claim 56.

67. A method for generating an antibody with a selected antimicrobial activity, comprising:

(i) providing a recombinant host cell population which expresses a soluble antibody library comprising a variegated population of test antibodies;

(ii) culturing the host cells with a target microorganism under conditions wherein the antibody library is secreted and diffuses to the target microorganism; and

(iii) selecting host cells expressing test antibodies that inhibit growth of the target microorganism.

68. The method of claim 67, wherein the target microorganism is a bacterium.

69. The method of claim 67, wherein the target microorganism is a fungus.

70. The method of claim 67, wherein the host cell is a bacteria.

71. The method of claim 67, wherein the host cells are cultured on agar embedded with the target microorganisms.

72. The method of claim 67, wherein the antimicrobial activity of the test antibody is determined by zone clearing in the agar.

73. The method of claim 67, wherein the antibody display library includes at least 103 different test antibodies.

74. The method of claim 67, wherein the test antibodies are 4-20 amino acid residues in length.

75. The method of claim 67, comprising the further step of converting into peptidomimetics, one or more test antibodies that inhibit growth of the target microorganism.

76. The method of claim 67 or 75, comprising the further step of formulating, with a pharmaceutically acceptable carrier, one or more test antibodies or peptidomimetics which inhibit growth of the target microorganism.

77. A method for preventing or treating infection of an animal by a microorganism, comprising administering to the animal a pharmaceutical preparation of claim 76.

78. A method for modulating an angiogenic process in an animal, comprising administering to the animal a pharmaceutical preparation of claim 46.

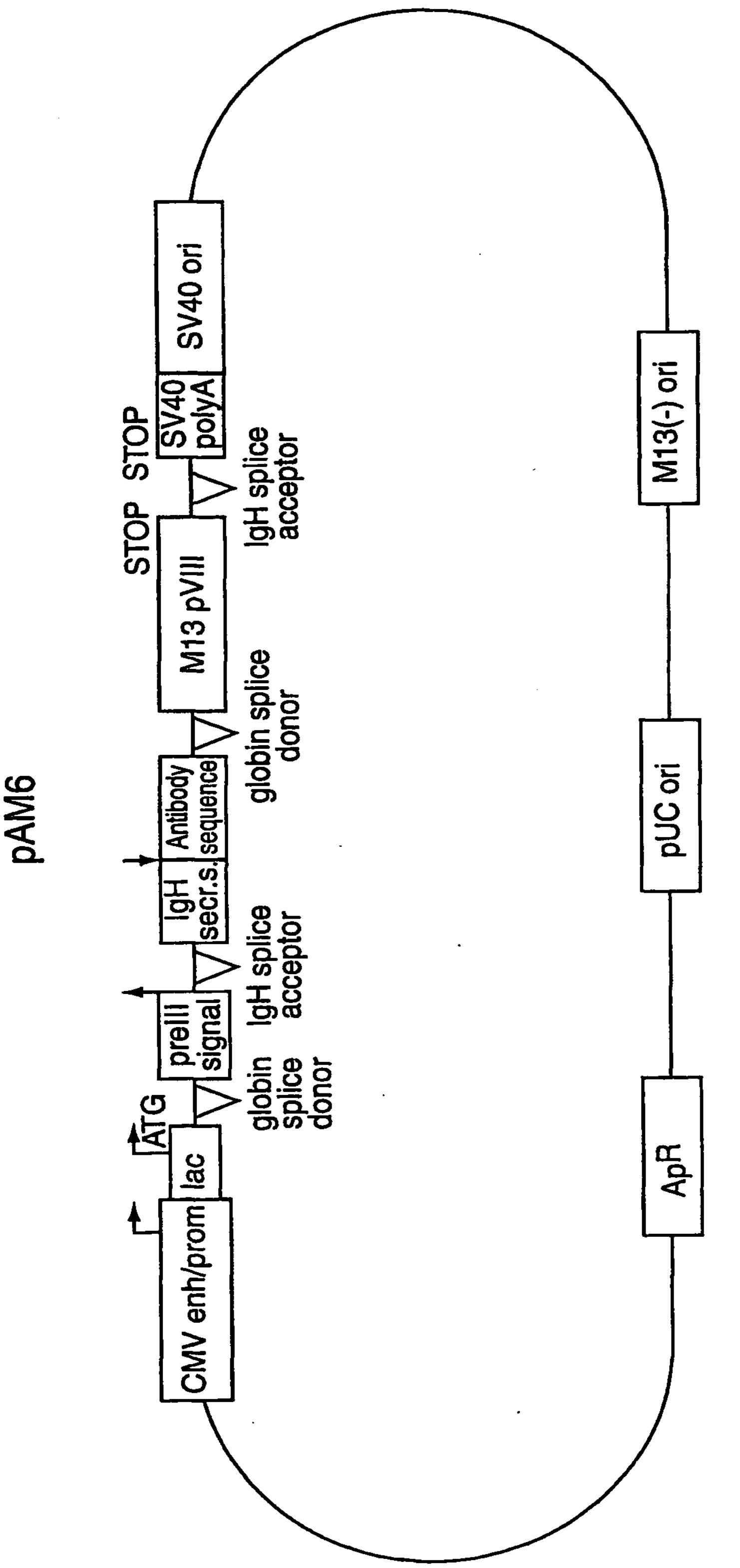
79. A construct as shown in Figure 1, 2, 3, or 4.

80. The method of claim 1, wherein the secreted test antibodies are dimerized.

81. The method of claim 25, wherein the chimeric gene further comprises (iv) a coding sequence for an antibody dimerization sequence, whereby the test antibodies dimerize upon secretion.

82. The vector of claim 48, wherein the chimeric gene further comprises (iv) a coding sequence for an antibody dimerization sequence, whereby the test antibodies dimerize upon secretion.

83. The vector library of claim 56, wherein the chimeric gene further comprises (iv) a coding sequence for an antibody dimerization sequence, whereby the test antibodies dimerize upon secretion.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CMV enh/prom
lac
ATG
globin splice donor
prelll signal
IgH splice acceptor
IgH secr.s.
Antibody sequence
globin splice donor
M13 pVIII
STOP
IgH splice acceptor
STOP
SV40 polyA
SV40 ori
ApR
pUC ori
M13(-) ori

Fig. 1

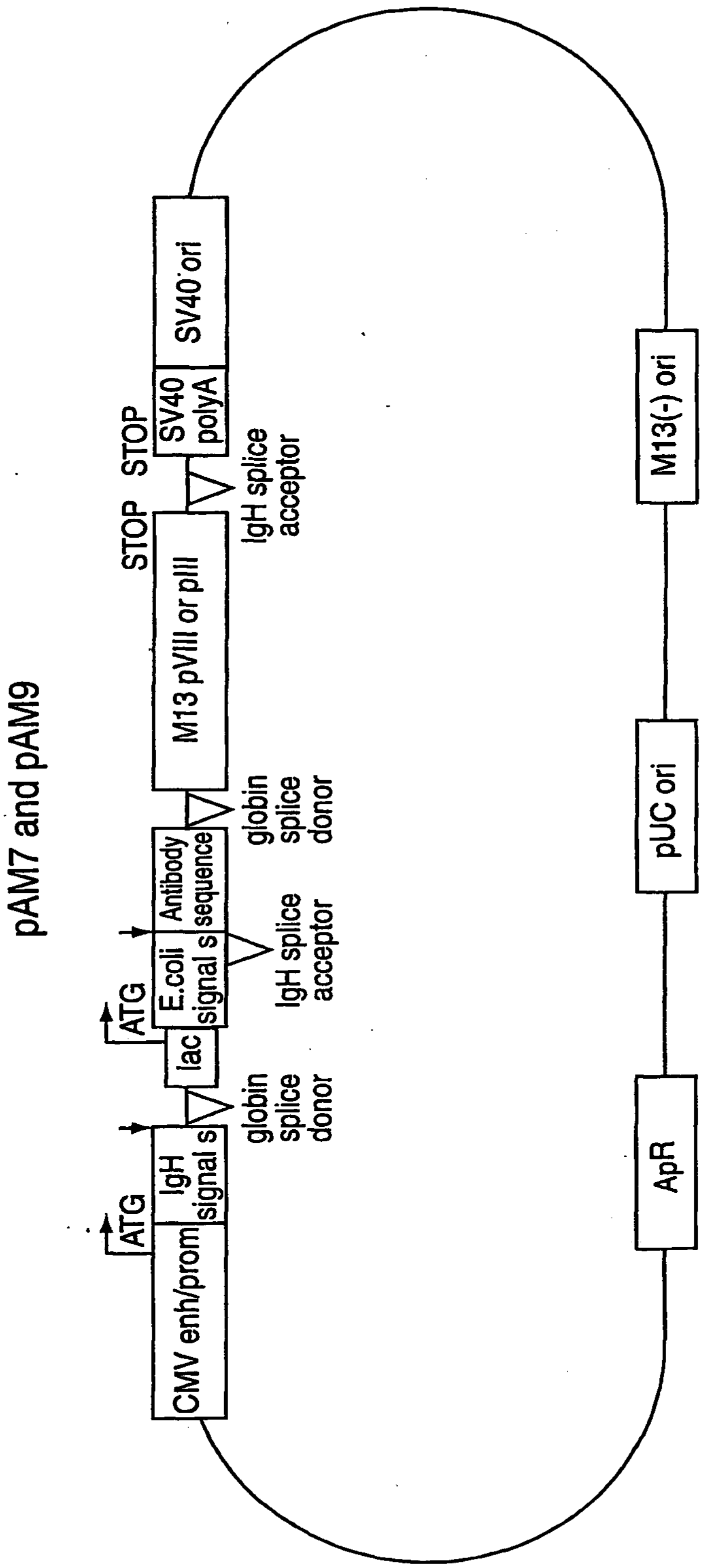
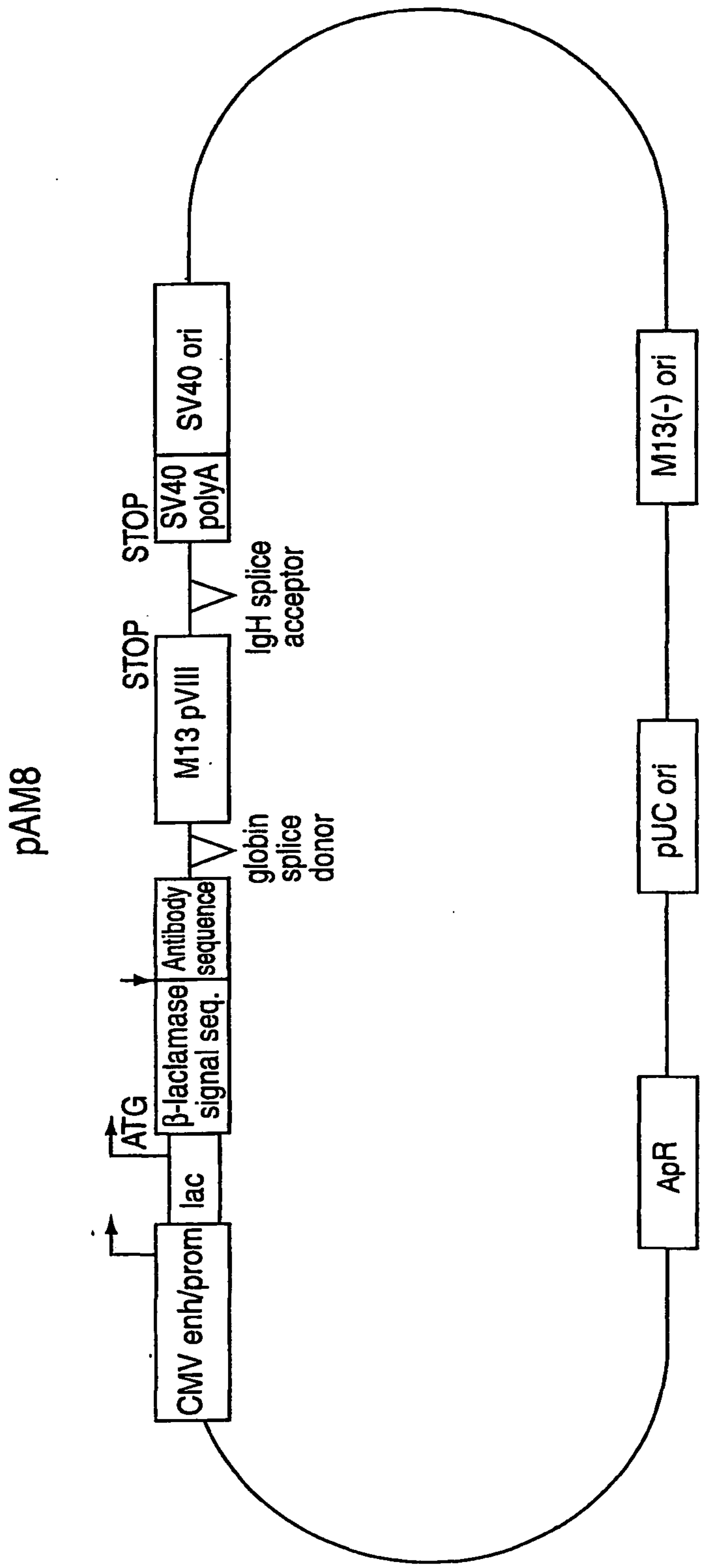

pAM7 and pAM9
CMV enh/prom
ATG
IgH signal s
globin splice donor
lac
ATG
E.coli signal s
IgH splice acceptor
Antibody sequence
globin splice donor
M13 pVIII or pIII
STOP
IgH splice acceptor
STOP
SV40 polyA
SV40 ori
ApR
pUC ori
M13(-) ori

Fig. 2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CMV enh/prom
lac
ATG
β-laclamase signal seq.
Antibody sequence
globin splice donor
M13 pVIII
STOP
IgH splice acceptor
STOP
SV40 polyA
SV40 ori
M13(-) ori
pUC ori
ApR

Fig. 3

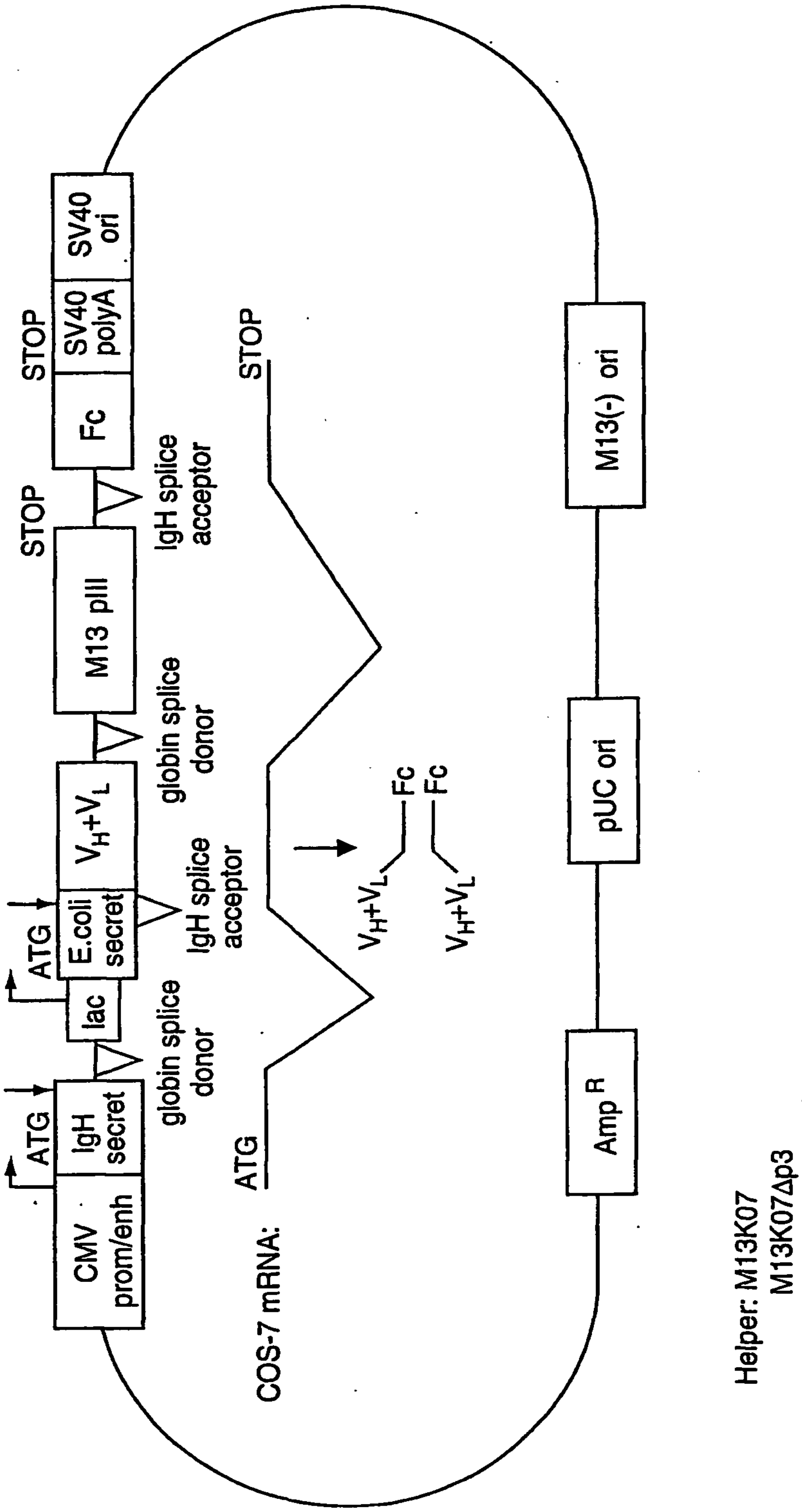
CMV
prom/enh
ATG
IgH
secret
globin splice
donor
lac
ATG
E.coli
secret
IgH splice
acceptor
V_H+V_L
globin splice
donor
M13 pIII
STOP
IgH splice
acceptor
Fc
STOP
SV40
polyA
SV40
ori
COS-7 mRNA:
ATG
STOP
V_H+V_L
Fc
V_H+V_L
Fc
Amp R
pUC ori
M13(-) ori
Helper: M13K07
M13K07Δp3

Fig. 4